



# Dedicated to B. K. Bachhawat on his 60th Birthday

## CONTENTS

Personal reflections .....	1
Mammalian sulfoconjugate metabolism .....	7
..... <i>Joy Mathew and A. S. Balasubramanian</i>	
Metabolism of an isolated brain perfused with perfluoro blood substitute. ....	23
..... <i>B. Mukherji and H. A. Sloviter</i>	
Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450. ....	35
..... <i>Minor J. Coon and Alfin D. N. Vaz</i>	
Oligosaccharide structure determination of glycoconjugates using lectins ....	41
..... <i>Debkumar Basu, Jyoti V. Nair and P. S. Appukuttan</i>	
Molecular biology of Ri-plasmid—A review .....	47
..... <i>Vilas P. Sinkar, Frank F. White and Milton P. Gordon</i>	
Mode of action of lipoic acid in diabetes .....	59
..... <i>S. S. Wagh, C. V. Natraj and K. K. G. Menon</i>	
How does cobalamin (vitamin B12) enter and traverse mammalian cells? ....	75
..... <i>Bellur Seetharam</i>	
Study of <i>Salmonella</i> endotoxin on the changes in lipid-protein interactions of membranes using Arrhenius plots of acetylcholinesterase as a tool .....	81
..... <i>M. K. Ray, A. Mitra, B. Mukherjee and G. C. Chatterjee</i>	
Definition of physical integrity of synaptosomes and myelosomes by enzyme osmometry. ....	89
..... <i>V. Sitaramam and M. K. Janardana Sarma</i>	
Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblast cells induced by glucocorticoids. ....	107
..... <i>Hasi R. Das, Robert Baratz and Delano V. Young</i>	
High affinity uptake of L-glutamate and $\gamma$ -aminobutyric acid in <i>Drosophila melanogaster</i> .....	119
..... <i>C. S. Ramarao, S. R. Acharya, K. S. Krishnan and U. W. Kenkare</i>	
Coimmobilization of D-amino acid oxidase and catalase by entrapment of <i>Trigonopsis variabilis</i> in radiation polymerised polyacrylamide beads .....	137
..... <i>Ashwini Deshpande, S. F. D'Souza and G. B. Nadkarni</i>	
Effect of <i>cis</i> and <i>trans</i> unsaturated fatty acids on the transport properties of <i>Salmonella typhimurium</i> .....	145
..... <i>I. K. Deb, B. Goyal and M. Chakravorty</i>	



RNase III	Hemanta K. Majumder, Sumit Adhya and Omadau Mitra	15
Transcriptional specificity after mycobacteriophage I3 infection	V. Nagaraja and K. P. Gopinathan	167
Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein via conjugative plasmid F in <i>Escherichia coli</i> K-12	Shambhavi Subbarao, Nam Prakash, A. V. Sivaprasad and Sushil Kumar	181
Stabilisation of some of the protein synthesis components in the thermophilic fungus, <i>Humicola lanuginosa</i>	Anil K. Joshi and Joseph D. Cherayil	193
Site-directed mutagenesis to determine essential residues of ribulose-bisphosphate carboxylase of <i>Rhodospirillum rubrum</i>	Salil K. Niyogi, Thomas S. Soper, Robert S. Foote, Frank W. Larimer, Richard J. Mural, Sankar Mitra, Eva H. Lee, Richard Machanoff and Fred C. Hartman	203
Purification and some properties of human DNA- <i>O</i> <sup>6</sup> -methylguanine methyltransferase	Amy M. Boulden, Robert S. Foote, G. Scott Fleming and Sankar Mitra	211
Autoregulation of gene expression: rho	Roberta Haber and Sankar Adhya	22
Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of <i>Vibrio cholerae</i>	Uma Bhattacharyya, Amit Ghosh and Ranajit K. Ghosh	23
RNase F and 2',5'-oligoA synthetase activities in mice after poly(I).poly(C) administration	Krishna Bakshi, Boris Skurkovich and Simon Skurkovich	23
Histochemical localization of protein-polysaccharides in renal tissue	S. Saraswathi, J. V. Tesoriero and N. S. Vasan	24
The positional specificities of the oxygenation of linoleic acid catalyzed by two forms of lipoxygenase isolated from Bengal gram ( <i>Cicer arietinum</i> )	Alip Borthakur, B. Ganesh Bhat and Candadai S. Ramadoss	25
Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase	K. Varalakshmi, H. S. Savithri and N. Appaji Rao	26
Multisubstrate specific amylase from mushroom <i>Termitomyces clypeatus</i>	Anil K. Ghosh and Subhabrata Sengupta	27
Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity	P. Shashidharan and L. K. Ramachandran	28
Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma	S. Mahadevan	29
Amphotycin: A tool to study protein N-glycosylation	Dipak Kumar Banerjee	31

Are the proteinase inhibitory activities in lenticular tissues real? .....	<i>S. Swaminathan and T. N. Pattabiraman</i>	321
Physicochemical properties and binding-site amino acid residues of galactoside-binding protein of human placenta. ....	<i>Madhusoodhan P. Nambiar, Debkumar Basu and P. S. Appukuttan</i>	331
Effect of chemical modification on structure and activity of glucoamylase from <i>Aspergillus candidus</i> and <i>Rhizopus</i> species .....	<i>B. C. Shenoy, A. G. Appu Rao and M. R. Raghavendra Rao</i>	339
Effect of alkaline pH on sunflower 11S protein .....	<i>G. Sripad and M. S. Narasinga Rao</i>	351
Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase $\alpha$ from embryonic chicken brain .....	<i>Ira Simet, Satyajit Ray and Subhash Basu</i>	361
A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver .....	<i>T. Chakraborty, I. B. Maiti and B. B. Biswas</i>	379
Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat. ....	<i>Nandini Nair and C. K. Ramakrishna Kurup</i>	391
Effect of mitogenic factors extracted from fetal lung fibroblasts on the <i>in vitro</i> growth of melanocytes obtained from normal and vitiligo subjects .....	<i>Manoj Mojamdar, Neelu Puri and Abburi Ramaiah</i>	399
Glycosaminoglycans in human fetal liver in relation to water and electrolytes .....	<i>Chameli Ganguly and K. L. Mukherjee</i>	409
Antigenic components of human glioblastoma multiforme and rat C <sub>6</sub> glioma using monoclonal antibodies. ....	<i>Ela Shamra, Kulvinder S. Kochhar and Shail K. Sharma</i>	423
Scavenging of superoxide radical by ascorbic acid .....	<i>Anuradha Nandi and I. B. Chatterjee</i>	435
Alteration of the acyl chain composition of free fatty acids, acyl coenzyme A and other lipids by dietary polyunsaturated fats. ....	<i>M. Renuka Prasad, Brenda Culp and William E. M. Lands</i>	443
Importance of determining viability of <i>Mycobacterium leprae</i> inside macrophages—an <i>in vitro</i> method using uracil .....	<i>Sunanda Vejare and P. R. Mahadevan</i>	455
<i>In vitro</i> effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases .....	<i>N. Giridharan, Mahtab S. Bamji and M. N. Madhyastha</i>	465
Conformational and ion-binding properties of cyclolinopeptide A isolated from linseed .....	<i>D. Chatterji, M. B. Sankaram and D. Balasubramanian</i>	473

Reactivity of glycoconjugate in membrane system II: can a neutral glycolipid function as lectin receptor in the presence of gangliosides in plasma membrane? S. K. Podder, A. Chakraborti, K. Vijayalakshmi and P. Lalit Kumar Singh	495
Mechanism of protein synthesis inhibition by vaccinia viral core and reversal of this inhibition by reticulocyte peptide chain initiation factors ..... Debopam Chakrabarti, Kolluru V. A. Ramaiah, Ananda L. Roy, Milan Bagchi ..... and Naba K. Gupta	503
Effect of proteolytic digestion on the function of vesicular stomatitis virus ribonucleoprotein complex ..... ..... A. K. Banerjee, J. Roy and D. J. Chattopadhyay	515
Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets Asok Banerjee, Sankar N. Maity, Sukla Ray Chaudhuri and B. Bhattacharyya	525
Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to <i>Vicia faba</i> lectin: Fluorescence-quenching studies ..... ..... Pradip K. Datta, Pranab S. Basu and Tapash K. Datta	537
Abnormal erythrocyte membrane phospholipid organisation in chronic myeloid leukaemia ..... ..... A. Kumar, S. Daniel, S. S. Agarwal and C. M. Gupta	543
Immobilization of <i>Kluyvera citrophila</i> penicillin acylase on controlled-pore ceramics ..... ..... A. M. Bodhe and C. SivaRaman	549
Conformational change of L7/L12 stalk in the different functional states of 50S ribosomes ..... ..... Debabrata Dash, Subodh Mahanti and Debi P. Burma	561
Arginine decarboxylase is a component activity of the multifunctional enzyme putrescine synthase in cucumber seedlings ..... ..... G. L. Prasad and P. R. Adiga	571
Subject Index .....	581
Author Index .....	589





B. K. Bachhawat (16th August 1925)

## Personal reflections

Dr. Bimal Kumar Bachhawat was born in August 1925. He got his B.Sc. Hons in Chemistry in 1946 from St. Xavier's College, Calcutta, and M.Sc. from the Department of Applied Chemistry, University of Calcutta, in 1948. He went abroad in 1949 for higher studies and obtained his Ph.D. from the University of Illinois in 1953 with biochemistry as the major. He began his post-doctoral career in USA first at Philadelphia and then as Assistant Professor at the University of Michigan at Ann Arbor.

Dr. Bachhawat returned to India in 1957 as Associate Professor of Neurochemistry, Christian Medical College, Vellore, where he founded one of the most active centres of biochemistry in India. His pioneering work on mucopolysaccharides in brain brought him world recognition and the coveted Shantiswarup Bhatnagar Award in 1962. This was followed by other awards such as the Amrut Mody Research Award in 1974 and the Institute of Science Golden Jubilee Gold Medal in 1976. He was elected a Fellow of the Indian National Science Academy in 1973, and the Indian Academy of Sciences, in 1974.

In 1976 Dr. Bachhawat was invited to take over as the Director, Indian Institute of Chemical Biology (then known as the Indian Institute of Experimental Medicine) and he held the position with distinction and honour upto 31st August 1985. After his retirement from the CSIR he was invited to join the Delhi University as Professor and Head of the Department of Biochemistry.

Among other awards and distinctions, Dr. Bachhawat was honoured with the R. N. Chopra Lectureship of the Indian National Science Academy, National Lectureship (1976) and J. C. Rose award of UGC (1980). Recently he received the prestigious Rameshwardas Birla Smarak Kosh Award for outstanding research in medical field in India.

Dr. Bachhawat's contribution to biochemistry and medicine has been characterized by a rare degree of width and versatility. His early work on the enzymes involved in branched chain amino acids led to the understanding of the linkage between metabolism of amino acids and steroidogenesis.

At Vellore he built an outstanding school of research to study the role of mucopolysaccharides in health and disease. His pioneering work on the deficiency of lysosomal arylsulphatase A in mental disorder and metachromatic leucodystrophy causing infantile mortality has been widely acclaimed.

Dr. Bachhawat also pioneered the work on liposomes, artificial membranes, which can be targeted to specific tissues with the help of specific markers. The liposome therapy, in which drugs, antibodies, enzymes and gene fragments could be delivered specifically to certain sites hold great promise in chemotherapy, immunotherapy as well as in some genetic disorders.

Dr. Bachhawat holds a unique distinction in blending brilliant science with superb leadership. He has had 30 Ph.Ds to his credit and about 175 research publications covering a wide spectrum of biosciences.

Among the few who claim to have the longest association with Prof. Bimal Kumar Bachhawat, I have been assigned the onerous task of writing a foreword to this volume of the Journal of Biosciences commemorating his sixtieth birthday. It would have been easier to present a chronological synopsis of his main achievements starting from his student days through his emergence as a leader in several areas upto his current role of a guiding light to diverse areas of biosciences. However, such an account would not be adequate to describe this unique personality.

I am, therefore, trying to present some of my personal impressions.

I met Bimal for the first time in 1950, when Prof. Carl Vestling brought him to my room at the University of Illinois at Urbana. Carl had told me earlier that he was expecting one B. K. Bachhawat from the Department of Applied Chemistry, University of Calcutta—the same Department that I came from. He also felt that after having about 4 year's seniority, I should be the proper guide to lead him through the complexity of the rehabilitation process in the campus. My first impression of Bimal was that he was a shy, polite, soft-spoken young man. But within a week, it was obvious that Bimal did not need any looking after and within a month he had just as many friends as I could make in the campus after 4 years.

Bimal started his research career with a bang! When Carl Vestling and Dave Gibson crystallized lactic dehydrogenase, the fourth enzyme to be crystallized from liver, Bimal was initiated into the witchcraft of enzyme purification by Carl, who had rare intuition about what methods to avoid. Bimal started soon on his main Ph.D. problem—the purification of liver malic dehydrogenase (MDH).

In 1950, the purification of an enzyme was an art rather than a science. The cellulose ion-exchangers, the molecular sieves and affinity chromatography techniques, which would make the task a trivial one, were 10–20 years ahead in the future. The “hocus pocus” that Bimal employed in the purification of MDH appeared to be childishly simple: (a) remove the junk proteins with ammonium sulphate and (b) precipitate the enzyme in a highly purified state with good yield with zinc and alcohol under highly controlled conditions. The fact that he got his Ph.D. in 1953, almost in a record time of two and a half years, shows that he did not have to move around in blind alleys—his “hunches” worked most of the time.

I have to relate an incident during his student days, an incident ingrained in my memory. Bimal and 5 of his colleagues were trying to move a 200 kg centrifuge into the cold room. The entrance was barely adequate and there was the wall of the deep freeze in front. It was taking a long time with awkward maneuvering and suddenly the centrifuge slipped from the hands of the person in front. But Bimal would not let go. The instrument slid on his patella. When Bimal was extricated, his leg started swelling. I would never forget the expression on his face. He was obviously in acute pain but he had also a triumphant smile. I had a new respect for him. He apparently valued the equipment more than his leg!

When he got his Ph.D., Bimal had already a growing reputation as a budding enzymologist. Minor Coon an alumnus of the Department of Biochemistry, University of Illinois, sought him out to work on metabolism of branched chain amino acids. In 1953 it was an exciting field—as the key intermediate to cholesterol and isoprenoid biogenesis was a puzzle. Bimal joined Minor Coon's team first as a post-doctoral associate at Philadelphia and then at the University of Michigan, Ann Arbor, as an

now ancient history. He purified quite a formidable number of enzymes in leucine and valine metabolism each purification bearing the hallmark of his "hocus pocus". During this period the most unusual achievement was the crystallization of what he calls "a functionless" enzyme. Years later a regulatory function has been assigned to his carbondioxide-ATPase. Similarly about 10 years later at Vellore he had again surprised the world by his purification of a "Substrate"—less enzyme Aryl sulphatase 'B'.

The chance discovery of "mevalonic acid" by Pete Tavormina (a mutual friend) at the E. Merck laboratory rather took away the carpet from the seekers of the key intermediate in isoprene biogenesis. It was time for Bimal to make a change.

When he returned to India in late 1957, Bimal already had a reputation as an upcoming biochemist and at the National Chemical Laboratory, we were hoping that he would join us. Jaganathan was frantically moving papers to create a suitable position for him. There were several other lucrative feelers. But Bimal surprised all of us by accepting a poorly paid position of an Associate Professor in the Department of Neurochemistry at the Christian Medical College, Vellore. At first his choice of Vellore puzzled us but now we know that the challenge of the problem of working in a difficult area—the biochemistry of myelination—the intricacy of the process of biogenesis of glycolipids attracted him more.

To realize the extent of his "audacity", one had to go back to the state of the art in 1957 in processing enzymes and membrane components. All selfrespecting biochemists used to avoid the field which required cumbersome, repetitive and often non-rewarding search for enzymes in intractable emulsions, gums and goos. But most biochemists in the world are now familiar with Bimal's work on mucopolysaccharide biogenesis and metabolism of gangliosides and cerebroside in some genetic disorders. Apart from his own impressive contributions, Bimal was able to establish one of the finest centres of biochemistry at Vellore, a Mecca for all biochemists.

However, Bimal had always a streak of scientific "wanderlust" in him. Instead of settling down on his laurels and achievements in the third phase of his life, suddenly he switched fields again. I distinctly remember my visit to Vellore in 1974 after about 3 years when everybody in his laboratory was talking about lectins and liposomes. Bimal told me that this switch was necessitated as he found that he had no funds and instead of complaining he was maintaining his laboratory by selling lectins. In 1975 it was an exciting game for him to introduce through liposomes with appropriate carbohydrate markers, all kinds of bizarre things into liver cells—things which should not be there.

In 1976 his acceptance of the Directorship of a CSIR organization "Indian Institute of Experimental Medicine" later to emerge in the scientific map of the world as the Indian Institute of Chemical Biology, was a pleasant surprise to his friends. All of them are used to Bimal's tenacity to do his highest thinking while having a meagre hand to mouth existence at Vellore. His initial salary of Rs. 500.00 was to continue for several years and then he had a larger existence as a Professor at Vellore for only Rs. 1,000.00 p.m.

His remarkable success as a builder and administrator was really amazing. The formidable task of revitalizing and rejuvenating required not only true scientific leadership but an unbelievable patience in sorting out non-scientific matters. The collection of some of the best young talents from India and abroad and to harness



the make the "dead wood" blossom into new buds.

To understand how Bimal achieved this miracle, let me describe a typical scene at the IICB when Bimal was Director. Bimal would be absorbed in a serious discussion with the administrative staff about construction of the animal house, installation of the purified water system and getting a date-line for the library air-conditioning when some irate senior scientist would suddenly barge in to complain about the non-availability of ordinary distilled water. He would be followed by a group of young students who want to have serious scientific discussion about their problems. By this time all the seats in the room have been taken and Sri Rabindra Singh, Bimal's personal attendant has seen to it that everybody present has at least half-a-cup of tea. Suddenly a group of 15 disgruntled professional trade unionists burst into his office demanding the same wages and social status as the scientists. What does one do under such a situation? Bimal fills his pipe with a wad of tobacco, lights it, takes a couple of puff for inspiration and then forgets about it. He talks to everybody, cuts some joke with each one. Only a few problems have been solved but each goes out smiling.

Bimal told me once that his secret of administration is to have no formal administration.

It is really unfortunate that Bimal had to retire when the achievements of the IICB was making their mark. The CSIR or any other organization in the country cannot officially recognize exceptional individuals. Even today the rules are more important than individuals. So his retirement was inevitable.

But from a broader point of view, there is a silver lining. Bimal's current occupation as a Professor in Delhi University is less likely to increase his hypertension than that of the Director of a CSIR laboratory. Now Bimal's friends and admirers are looking for the beginning of the Sixth Phase of his life—creation of more new science and new tradition.

My life has been enriched by having Bimal as a friend and philosopher-and-even though I am older I am not ashamed to gratefully acknowledge his guidance not only in science but also in other problems. Bimal is a remarkable person whose love for science transcends into a love for fellow human being. Although not religious, he typifies the highest principles of Jainism—love, compassion and supreme detachment.

It will be meaningless to wish merely a long life for him. It will be more appropriate to wish him to remain as the shining light to guide all adventurers into the realm of biosciences for many more years to come.

P. K. Bhattacharyya  
Indian Institute of Chemical Biology  
Calcutta

### III

#### *Pleasure and privilege*

The title of the statement to follow was selected after some thought about the career to date of our Honoree. Please note the underline which clearly denotes that Bimal is

Biochemistry at the University of Delhi. I refer to him as Bimal without his permission as you see.

It is a privilege for me to be invited to participate in this well deserved celebration. It is also a real personal pleasure for me to comment on the distinguished career of a graduate of the University of Illinois at Urbana, Illinois (Ph.D., 1953). It was both a privilege and a pleasure for me when Bimal elected to do his Ph.D. thesis studies in my laboratory. I have followed his career in a very general way and have been aware of his major impact not only on the scientific aspects of his productive research programs but also on his personal interactions at all levels (students, faculty, government, national and international meetings and the like). Bimal is a true example of the universality of good science and of the continuing challenges to our understanding. This latter point is relevant in spite of the remarkable and explosive leaps forward in biological science during the past 25 years or so.

Let me ask two questions: What exactly can a young graduate student in a major field of study hope for and expect as he pursues his course work and Ph.D. thesis preparation? At the same time what exactly can a graduate student's mentor hope for and expect? The eyes of both are on the specifics of formal and informal education and on the vastness of established knowledge and of the partially explored and unexplored vistas ahead. Graduate study is an exciting time, but it is really only a preparation for future experiments and analysis. It has been a continuing privilege and pleasure for me to observe how a good, active and industrious graduate student can move logically and steadily into his important career. Bimal's example is a strong one indeed.

This account is being written on the shores of Lake Michigan, about 2100 miles from my library. I am armed with Bimal's Curriculum Vitae and with a copy of the abstract of his Ph.D. thesis, entitled 'The Purification of Malic Dehydrogenase from Rat Liver'. I must state that the University of Illinois Library, one of the great libraries in the USA, has lost the original first typed copy of his thesis (no Xeroxing in 1953) and is now making a strong effort to locate it. My personal copy is safely in my study and is not easily available to me because of time constraints related to the publication deadline. What I would like to do is to point out the state of enzyme isolation, purification and characterization then (1953) and now (1986) as shown by Bimal's thesis.

Bimal's first major paper was as a co-author with Dr. David M. Gibson (M.D., Harvard, and postdoctoral fellow in our laboratory), E. O. Davisson (graduate student), Dr. B. R. Ray (Professor of Physical Chemistry) and myself. [Rat Liver Lactic Dehydrogenase. I. Isolation and Chemical Properties of the Crystalline Enzyme, *J. Biol. Chem.*, **203**, 397-409 (1953)]. Suffice it to say two things: Our lactate dehydrogenase (LDH) was the fourth crystalline enzyme from mammalian liver (after catalase, alcohol dehydrogenase, and glutamic dehydrogenase). This result came after more than two years of intensive effort to fractionate liver homogenates. We were at the stage of fractional salting out, low temperature ethanol precipitation at controlled ionic strength and a variety of hopeful efforts to gain in enzymatic specific activity without losing too much of it. Bimal jumped in and helped in what turned out to be a successful effort.

As a thesis project I proposed to Bimal that he devote himself to the liver supernatant phase following 50% saturation with ammonium sulphate. This step

precipitated all the LDH and apparently left behind in solution the malic dehydrogenase [now malate dehydrogenase (MDH)]. At that time we were not aware of the existence of both mitochondrial and cytosolic MDH's (to be announced considerably later by H. A. Lardy and co-workers). I recall that Bimal and I agreed that several week-ends of work would likely lead us to gram quantities of pure MDH to be characterized and studied in terms of kinetic mechanism. As events developed it was clear that we were overly optimistic, and Bimal undertook a careful study of protein separation techniques which led to a highly purified—but not very stable—MDH preparation. He introduced some new approaches, including low ionic strength-ethanol extraction of liver homogenates and zinc precipitation techniques (after E. J. Cohn and co-workers, with the use of  $^{65}\text{Zn}$  as a tracer). Bimal also developed a new assay for glutamate-oxalacetate transaminase (now aspartate aminotransferase) by coupling it with the MDH-catalyzed reduction of oxalacetate by NADH. This useful procedure has been widely used since that time. We sent it off to a major journal for publication as a communication but were told that we should use the method further in connection with proposed studies of transaminase. As a result other investigators published the procedure before we were ready. To summarize: Bimal produced a thoughtful thesis with a lot of careful experimentation.

In this connection it can be mentioned that only very recently was cytosolic MDH from liver crystallized and subjected to X-ray structural studies. Mitochondrial MDH turned out to be exceedingly difficult to crystallize in our hands, and we were unable to establish thoroughly the function of tightly bound lipid(s) before we closed our laboratory in 1981. So Bimal's and my "several week-ends" grew into a career-long effort. The extent of progress in protein isolation can be illustrated by our recent use in a class experiment (undergraduate biochemistry majors) of a pseudo-affinity chromatographic column technique (Blue Affi-Gel, Biorad) which allows the recovery of highly purified LDH from chicken breast muscle in about 2 h.

Following Illinois Bimal spent two postdoctoral years at Pennsylvania and then two more, at Michigan before returning to India. Let me say that his career in India can be looked at most definitively by scanning through the titles of his more than 151 papers, most of which are "full papers" and not abstracts of presentations at scientific meetings. He has clearly met the challenge of keeping up with and developing new approaches with sound and innovative enzymology as a recurring theme. While probing neurochemical matters he has studied glycoproteins and other conjugates and has turned to the application of new knowledge to medical and diagnostic problems. He has focused on the study of the role of glycoproteins as cell surface receptor sites and joined the large number of investigators of cell receptors.

Most of all Bimal has been true to his science, to his family, his colleagues and his country, and to himself. The publication of tributes to him brings pleasure to all of us for the privilege of working with him and honoring him at this most active stage of his career in Biochemistry.

Carl S. Vestling  
University of Arizona  
Tucson, Arizona, USA

# Mammalian sulfoconjugate metabolism

JOY MATHEW and A. S. BALASUBRAMANIAN

Neurochemistry Laboratory, Department of Neurological Sciences, Christian Medical College and Hospital, Vellore 632 004, India

**Abstract.** Sulfoconjugates occur ubiquitously as sulfopolysaccharides, sulfolipids and sulfoproteins. A variety of sulfotransferases catalyze the sulfation process with 3'-phosphoadenosine 5'-phosphosulfate as the sulfate donor. Sulfatases that catalyze the desulfation of different sulfoconjugates are known to be deficient in a number of genetic storage disorders.

**Keywords.** Sulfoconjugates; sulfotransferases; sulfatases; genetic disorders.

## Introduction

Numerous reviews of this subject have been published dating as far back as 1945 (Lugg, 1945). In recent years, there has been wide-spread interest in the study of sulfoconjugates and their metabolism (for selected reviews, see Gregory and Robbins, 1960; Roy, 1960a; Balasubramanian and Bachhawat, 1970; Dodgson and Rose, 1970, 1975; Farooqui, 1980, 1981). This article deals with the more recent aspects.

The importance of sulfur containing compounds stems from the fact that they participate in virtually all living processes. It is present in organisms as constituents of proteins, coenzymes and as major cellular metabolites like glutathione. Although in some cases, sulfate esters may serve no physiological role other than sulfur storage *e.g.* choline O-sulfate), the sulfated glycosaminoglycans of connective tissue or the sulfatides of the nervous system and most cell membranes are excellent examples of their structural function. Protein sulfation of tyrosine residues has been found to occur in most cell types in culture and in many tissues (Huttner, 1982). The recent discovery of a large number of secreted sulfoproteins in plasma (Hille *et al.*, 1984) and neuronal axons (Stone *et al.*, 1984) and the finding that sulfoproteins meet critical requirements for consideration as secretable fast-transported proteins has generated tremendous interest in the study of sulfate metabolism and related enzymes. Further, the drastic reduction in tyrosine O-sulfated proteins in rat fibroblasts in culture infected with Rous sarcoma virus (Liu and Lipman, 1984) in contrast to the increase of tyrosine O-phosphate in a number of malignantly transformed cells are indicative of a transformation mediated change in sulfoprotein level. The sulfated enkephalins present in brain (Unsworth *et al.*, 1982) may act as an inactive pool of enkephalins while the sulfation of neural cell adhesion molecule may have a role in its neurone-neurone and nerve-muscle adhesions (Sorkin *et al.*, 1984). Sulfoconjugation may result in the masking of the physiological activity of certain compounds (*e.g.* steroid hormones, vitamin D, etc.) or serve as a detoxification mechanism (sulfoconjugation of phenols). On the other hand, the presence of cholesterol sulfate in the plasma membrane of spermatozoa may help in sperm capa-

resists enzymatic deglycosylation much like the placental gonadotropins which are protected by peripheral sialic acids.

The discovery of human genetic disorders of sulfate metabolism has led to a major boom in the research on the enzymes that are involved in the synthesis (sulfotransferases) and degradation (Sulfo-hydrolases) of sulfated compounds.

### *Sulfotransferases*

Sulfotransferases catalyze the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate (active sulfate or PAPS) to a suitable acceptor containing a phenolic hydroxyl group. There are a variety of sulfotransferases present in animal system. The most widespread among them are arylsulfotransferases present in the soluble fraction of cell preparations where the enzymatic system responsible for the biosynthesis of PAPS exists. Of the arylsulfotransferases, phenolsulfotransferase, steroid sulfotransferase and tyrosylprotein sulfotransferase have been well studied in mammalian system.

### *Phenolsulfotransferases*

The presence of phenolsulfotransferases has been demonstrated in brain, liver, kidney, adrenals, jejunum (Bostrom and Wengle, 1967) and platelets (Hart *et al.*, 1979) of man. There are two types of phenolsulfotransferases designated the P-form which sulfates exogenous phenolic compounds and the M-form that sulfates endogenous phenolic substances such as monoamines (Rein *et al.*, 1982). Both forms were present in human brain and platelets (Young *et al.*, 1985). The P-form of the enzyme may have a role to play in neutralizing dietary phenolic compounds as patients with dietary migraine had significantly lower levels of platelet phenol-sulfotransferase activity than either migrainous patients without a history of dietary provocation or normal controls. The P-form of the enzyme was more severely involved than the M-form (Littlewood *et al.*, 1982). It is suggested that the primary function of both forms of phenolsulfotransferase in brain may not be connected with monoamine metabolism, but may primarily be to protect the brain from low circulating levels of potentially noxious dietary phenols (Rein *et al.*, 1984). Recently Whittemore *et al.* (1985) purified the M-form of phenolsulfotransferase from human brain to about 20,000 fold. The enzyme had a molecular weight ( $M_r$ ) of 250,000 by gel filtration and it had no phenolsulfating activity. 4-Methoxytyramine was the most preferred substrate. A similar enzyme purified to 272-fold from human brain cortex (Yu *et al.*, 1985) had a  $M_r$  of 62,000 and high affinity towards dopamine and m-tyramine. It was moderately active towards noradrenalin and p-tyramine while serotonin was a poor substrate.

### *Steroid sulfotransferases*

This group of enzymes catalyze the sulfation of alcoholic hydroxyl groups. Several of them are known that sulfate estrone, estradiol, testosterone, androstenediol, dehy-

cholesterol (Zim and Fierowitz, 1966). Recently, Tseng *et al.* (1985) have partially purified a human placental estrogen sulfotransferase. It had a pI of 5.8 and  $M_r$  of 68,000 on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Even though the enzyme could sulfate estrone, estriol and dehydroepiandrosterone, estradiol was the best substrate. The effect of androgens and estrogens on rat hepatic bile acid sulfotransferase has been investigated by Kirkpatrick *et al.* (1985). The former had a suppressing effect while estrogens stimulated the sulfotransferase. Consequently, female rats had a 3-fold higher activity than males (Chen and Thaler, 1984). Similar effects were observed on a sulfotransferase described in rabbit uterine endometrium (Munakata *et al.*, 1985). Recently, Chen and Segel (1985) purified a bile salt sulfotransferase from human liver to about 760-fold and showed it to be an -SH enzyme with a pI of 5.2 and  $M_r$  of 67,000. The sulfotransferase had no activity towards estrone, testosterone or phenol.

### *Tyrosylprotein sulfotransferase*

Even though the presence of tyrosylprotein sulfotransferase had been demonstrated as early as 1966 (Banerjee and Roy, 1966), the real interest was awakened only recently when Huttner (1982) demonstrated the protein sulfation on tyrosine residues of virtually all cell lines and tissues. This was followed up by a large number of investigators unraveling the secretory nature of most sulfoproteins. A tyrosylprotein sulfotransferase has been described by Lee and Huttner (1985) in the Golgi membrane fraction of a bovine adrenal medulla homogenate. The enzyme could sulfate tyrosine residues of tubulin in the presence of a nonionic detergent. A polymer, (glutamyl, alanyl, tyrosinyl) served as a high affinity substrate. A similar enzyme has been described by Vargas *et al.* (1985) in a crude microsomal fraction from rat cerebral cortex. Various cholecystokinin fragments or derivatives could act as sulfate acceptors. Peptides with an N-terminal tyrosine residue (*e.g.* enkephalins or CCK-7) were not sulfated. The importance of these sulfoproteins are yet to be worked out. Other than as secretory proteins, they may be involved in the overall function of the protein in an identical manner as phosphoproteins.

Sulfotransferases such as choline sulfotransferases are involved in the sulfation of choline, dimethylaminoethanol and dimethylethylaminoethanol (Fitzgerald, 1973; Kaji and Gregory, 1959; Nissen and Benson, 1961). There are sulfotransferases that sulfate aromatic amines such as aniline or 2-naphthylamine (Roy, 1960b). In rat hepatocytes sulfation of the phenolic hydroxyl group of iodothyronines facilitate the deiodination of these compounds (Otten *et al.*, 1983). Sulfolipid sulfotransferases are present in the brain (Mckhann *et al.*, 1965; Balasubramanian and Bachhawat, 1965; Cumar *et al.*, 1968), kidney (Fleischer and Smigel, 1978) and testes where it is involved in spermatogenesis (Lingwood, 1985). The rat kidney galactocerebroside sulfotransferase has been purified to apparent homogeneity. It was hydrophobic in nature with a  $M_r$  of 64,000 and pI of 5.1 with essential arginine residues at the active site (Tennekoon *et al.*, 1985).

Different sulfotransferases are present for the sulfation of glycosaminoglycans resulting in the formation of chondroitin 4- and 6-sulfates, heparan sulfate (Suzuki *et al.*, 1961) and dermatan sulfate (Hasegawa *et al.*, 1961). Balasubramanian and Bachhawat (1964) demonstrated the presence of a mucopolysaccharide sulfotrans-

ferase in rat brain cytosol that could sulfate a variety of substrates. Habuchi and Miyashita (1982) separated chondroitin 4- and 6-sulfotransferase from 14-day old chick embryos by gel filtration and showed that addition of polyamines and basic proteins to the incubation medium had an activating effect on both transferases.

Investigations by various workers (Young, 1973; Richmond *et al.*, 1972, 1973; DeLuca *et al.*, 1973) have localized the glycosaminoglycan sulfotransferases on the Golgi and concluded that sulfating enzymes were located together with polymerizing enzymes in an enzyme complex so that sulfation of the heteropolysaccharide chain proceeded during or immediately following polymerization. Two keratan sulfotransferases have been partially purified from isolated bovine corneal cells and shown to have a  $M_r$  each of 220,000 and 140,000, respectively. The preparation did not show any chondroitin sulfotransferase activity and both enzymes acted in a co-operative manner on keratan sulfate fragments, which was the best preferred substrate (Rueter and Kresse, 1984). Evidence for the recognition of the N-acetylgalactosamine residues in the non-reducing terminal regions of oligosaccharides by the transferases was revealed by Delfert and Conrad (1985) while working with microsomal preparations from cultured chick embryo chondrocytes which showed a high rate of sulfation at the reducing ends of shorter oligosaccharides, but decreased with increasing chain length.

On the whole, sulfotransferases are involved in the synthesis of all the biologically occurring sulfoconjugates and thus participate in the overall functioning of the system.

### *Sulfohydrolases*

The class of enzymes that hydrolyze esters of sulfate and related sulfoconjugates are collectively called sulfohydrolases or sulfatases. The role of sulfatases is profound in maintaining the concentrations of various sulfoconjugates in physiological fluids or the turnover of sulfated compounds in tissues, in cell-mediated cytolysis or in the breakdown of dietary sulfate esters. If the recent developments in the study of sulfated cell surface glycosaminoglycans (Wieland, 1982; Dietrich, 1984; Hook *et al.*, 1984) are any indication, sulfohydrolases have a very important role to play in cell growth and cell division.

Sulfatases may be generally classified into alkyl, aryl and carbohydrate sulfatases.

### *Alkylsulfatases*

Sulfatases that hydrolyze the sulfate esters of simple alcohols such as methanol, ethanol, etc. are termed as alkylsulfatases. Although the formation of alkylsulfate esters in mammalian tissues is well known (Vestermarck and Bostrom, 1959; Spencer, 1960), the corresponding hydrolases have not been described so far except a choline sulfatase in higher fungi and some bacteria.

Steroid alkylsulfatases such as dehydroepiandrosterone sulfatase, a microsomal enzyme rich in placenta and testes (French and Warren, 1967) are widely known. Sulfatases that hydrolyze sulfate esters of estrone, pregnenolone, testosterone, cholesterol and *p*-nitrophenol have been described in rat brain (Iwamori *et al.*, 1976a,b).

Steroid sulfatase is involved in the mechanism of sperm capacitation and ovum penetration (Langlais *et al.*, 1981). Studies by Ropers and Wieberg (1982) suggested that the steroid sulfatase activity in cultured cells of wood lemming is directly correlated to the number of X-chromosomes and that the sulfatase gene is also X-linked and not subject to inactivation while in mice the steroid sulfatase gene is subject to the normal pattern of X-inactivation (Crocker and Craig, 1983).

The insoluble nature of steroid sulfatase had hampered attempts at purification until Noel *et al.* (1983) and Burns (1983) purified the enzyme 100- and 300-fold, respectively from human placental microsomes. The preparation obtained by Noel *et al.* (1983) was heterogeneous in nature and it could hydrolyze sulfate esters of cholesterol, dehydroepiandrosterone and *p*-nitrophenol while the sulfatase purified by Burns was homogeneous and hydrolyzed dehydroepiandrosterone sulfate and *p*-nitrophenylsulfate. Both preparations exhibited a  $M_r$  range of 72–74,000 daltons.

There is evidence to suggest that different sulfatases are responsible for the hydrolysis of the sulfate esters of estrone, dehydroepiandrosterone, cholesterol and *p*-nitrophenol (Eto *et al.*, 1974; Mathew and Balasubramanian, 1982; Milewich and Garcia, 1985; Prost *et al.*, 1985). Further Simard *et al.* (1985) have separated steroid sulfatase and arylsulfatase C (4-methylumbelliferyl or *p*-nitrophenylsulfatase) by cellogel electrophoresis and demonstrated that they are immunologically different. It is possible that steroid sulfatase could hydrolyze the artificial substrates of arylsulfatase C and not *vice versa* (Horwitz *et al.*, 1986; Simard *et al.*, 1985). Steroid sulfatase is stimulated by phosphatidylcholine (McNaught and France, 1980). The significantly lower level of this lipid in steroid sulfatase deficient placenta (McKee and France, 1983) suggest that phosphatidylcholine may have a role to play in steroid sulfatase activity.

### *Arylsulfatases*

Arylsulfatases are the most widely distributed of all the sulfohydrolases. The arylsulfatase present in natural killer cells are involved in the lysis of the target cell membrane (Zucker-Franklin *et al.*, 1983). They are classified into arylsulfatase A, B and C. The first two are soluble lysosomal enzymes and they are inhibited by phosphate and sulfate while arylsulfatase C is an insoluble microsomal enzyme which is not affected by phosphate or sulfate. Many of the arylsulfatases have been purified and identified (table 1).

*Arylsulfatase A:* Arylsulfatase A is perhaps the best studied of all the arylsulfatases. It has been purified from a variety of sources by a large number of workers (Hess, 1983; Waheed and Van Etten, 1985a; Laidler *et al.*, 1985; Nakamura *et al.*, 1984). Sulfatase A is an acidic glycoprotein (Balasubramanian and Bachhawat, 1975) with a pI of 4.6–4.8 (Eto *et al.*, 1974). It has a monomeric  $M_r$  of 102,000–107,000 and a carbohydrate content of 15–25%. The enzyme is rich in proline, aspartic and glutamic acids and hydrolyzes sulfate esters such as cerebroside 3-sulfate, seminolipid, psychosine sulfate, ascorbic acid 2-sulfate and tyrosine O-sulfate (Farooqui, 1980).

The arylsulfatase A purified from human liver (James and Austin, 1979) contained two subunits of  $M_r$  69,000 and 57,000 which on further studies proved similar sugge-



Table 1. Properties of arylsulfatases.

	Arylsulfatase A	Arylsulfatase B	Arylsulfatase C
Localization	Lysosomal	Lysosomal	Microsomal
Nature	Glycoprotein	Glycoprotein	Glycoprotein
Isoelectric point	3.8	8.0	8.1
Molecular weight	100,000	38,000	280,000
Sedimentation coefficient	6.5	4.5	4.85
Kinetics	Abnormal	Normal	Normal
pH optimum	Acidic	Acidic	Alkaline
Effect of sulfate	Competitive inhibition	Non-competitive inhibition	No effect
Effect of cyanide	No effect	No effect	Inhibition
Effect of silver	Marked inhibition	Slight stimulation	Inhibition
Synthetic substrates	Nitrocatechol sulfate	Nitrocatechol sulfate	<i>p</i> -nitrophenyl sulfate
	Methylumbelliferyl sulfate	Methylumbelliferyl sulfate	<i>p</i> -acetylphenyl sulfate
	Cerebroside 3-sulfate	UDP-N-acetylgalacto-	Steroid sulfates?
	Tyrosine O-sulfate	samine 4-sulfate	
	Ascorbic acid 2-sulfate		
	Lactosyl sulfate		
	Sulfogalactosyl sphingosine		
	Sulfogalactosyl glycerolipid		
Natural substrates			

Waheed and Van Etten, 1985b) and had essential arginine residues (James, 1979). It exhibits abnormal kinetics and the activity is linear only upto 10–15 min due to a substrate induced inactivation of the enzyme, which is accompanied by the binding of a sulfate ion from the substrate (Prosser and Roy, 1980; Waheed and Van Etten, 1980) followed by reactivation by sulfate in the presence of the substrate. Roy (1985) described arylsulfatase A as a hysteretic enzyme and proposed a progress curve for the reaction catalyzed by the enzyme.

An atypical arylsulfatase purified from chicken brain (Farooqui and Bachhawat, 1972) hydrolyzed cerebroside 3-sulfate, but had properties of both arylsulfatase A and B. The possibility of a single enzyme hydrolyzing the substrates of both arylsulfatase A and B cannot be ruled out as only a single arylsulfatase was present in chicken brain (Mathew and Balasubramanian, 1984).

Das and Bishayee (1980) separated sheep brain arylsulfatase A into high and low-uptake forms. The former was susceptible to alkaline phosphatase indicating the presence of phosphate residues. The enzyme purified from human liver exhibited molecular heterogeneity (Sarafian *et al.*, 1984). Sialic acid residues were partly responsible as neuraminidase treatment could bring down the heteromer bands to only 3. Similar studies in lung tumors (Nakamura *et al.*, 1984) and bladder cancer patients (Mitsunashi *et al.*, 1984) suggest an increased level of microheterogeneity of arylsulfatase A in transformed tissues. Recently, (Waheed and Van Etten, 1985b) demonstrated the variations in carbohydrate content of arylsulfatase A in normal and carcinoma cell lines and concluded that the carbohydrate free enzymes were essentially similar with a  $M_r$  of 59,000 daltons and attributed this to an elevated level of glucosyltransferase.

**Arylsulfatase B:** Arylsulfatase B is similar to arylsulfatase A in being a soluble lysosomal glycoprotein exhibiting molecular heterogeneity (J. Mathew and A. S. Balasubramanian, unpublished results). It is cationic in nature and has been purified from a variety of sources (Agogbua and Wynn, 1976; Farooqui and Roy, 1976; McGovern *et al.*, 1982; Weller and Austen, 1983). Multiple forms of arylsulfatase B has been reported from ox tissues (Bleszynski *et al.*, 1969), human placenta (Gniot-Szulzycka, 1972), human brain and liver (Harzer *et al.*, 1973) which can be separated by chromatographic and electrophoretic methods. The nature and significance of these multiple forms are not clear at present even though sialic acid (Harris *et al.*, 1982), phosphate residues (Uehara *et al.*, 1983) and varying carbohydrate content have been implicated (Farooqui and Roy, 1976).

Sulfatase B from ox liver and brain is shown to be rich in proline and basic aminoacids. The enzyme has large amounts of tyrosine and an essential histidine residue in its active site. The ability of arylsulfatase B to hydrolyze UDP-N-acetylgalactosamine 4-sulfate was first demonstrated by Fluharty *et al.* (1975) and later confirmed by using homogeneous preparations of arylsulfatase B (Farooqui, 1976). The enzyme could also hydrolyze glucosamine 4,6-disulfate, but not glucosamine 6-sulfate.

A minor anionic form of arylsulfatase B present in significant amounts only in brain, but not in other tissues like liver, kidney, testes and placenta was isolated by

purified from monkey brain had a  $M_r$  of 30,000 and a phosphate content of about 7.0 mol of inorganic phosphorus per mol of protein (Lakshmi and Balasubramanian, 1984).

Arylsulfatase B is located on chromosome 5 as compared to chromosome 22 of arylsulfatase A and the fact that each enzyme is expressed independently provides evidence against any common structural feature between the two enzymes (DeLuca *et al.*, 1979).

Cancer associated modification of arylsulfatase B has been reported in lung tumors (Gasa *et al.*, 1981) which is speculated to be due to transformation mediated elevation of sialyltransferase and protein kinase (Niinobe *et al.*, 1979; Kottgen *et al.*, 1978) resulting in the phosphorylation of arylsulfatase B both on its protein and carbohydrate moiety (Gasa and Makita, 1983). Human brain anionic B was taken up rapidly by multiple sulfatase deficient fibroblasts whereas arylsulfatase B was practically not taken up into the cells. Alkaline phosphatase treatment could abolish this high-uptake activity without diminishing its catalytic activity (Kureha and Eto, 1983).

Steckel *et al.* (1983) showed that arylsulfatase B synthesized and secreted as a 64,000  $M_r$  precursor was processed to a 62,000 dalton intermediate and mature forms of  $M_r$  47,000, 40,000 and 31,000 daltons. The processing of 62,000  $M_r$  intermediate to the pair with  $M_r$  40,000 and 31,000 depended on cysteine proteinases (Steckel *et al.*, 1985).

**Arylsulfatase C:** The presence of arylsulfatase C was first demonstrated by Dodgson *et al.* (1954) in rat liver microsomes and later in various human tissues (Dodgson *et al.*, 1956). The enzyme is located in the endoplasmic reticulum and the nuclear outer membrane (Zemelman *et al.*, 1985). The numerous similarities observed between mammalian arylsulfatase C and steroid sulfatase had led to the hypothesis that a single enzyme is responsible for both activities (Dolly *et al.*, 1972; Balasubramanian, 1976; Iwamori *et al.*, 1976b). The chaotropically solubilized arylsulfatase C from monkey brain has been shown to require a dialyzable activator which had no effect on the estrone sulfatase activity from the same source (Lakshmi and Balasubramanian, 1979). This led to the possibility of separate enzymes responsible for the hydrolysis of *p*-nitrophenylsulfate and estrone sulfate. Making use of the hydrophobic nature of arylsulfatase C, it could be partially separated from estrone sulfatase by hydrophobic interaction chromatography (Mathew and Balasubramanian, 1982).

Persistent attempts by workers to purify arylsulfatase C were unsuccessful until Moriyasu *et al.* (1982) obtained a homogeneous preparation from rat liver microsomes. The native enzyme had a  $M_r$  of 280,000 by gel filtration while the monomeric  $M_r$  was 72,000. It was a glycoprotein with a pI of 8.1. The carbohydrate part of the enzyme was rich in mannose and N-acetylglucosamine. It is not known whether steroid sulfatase activity is associated with this preparation.

The physiological substrate of arylsulfatase C is not known. The enzyme purified from placenta (Noel *et al.*, 1983; Burns, 1983) has steroid sulfatase activity. Arylsulfatase C purified from human skin fibroblasts (Simard *et al.*, 1985) exhibited two immunologically different bands on cellogel electrophoresis. Both forms hydrolyzed

A large number of carbohydrate sulfate esters such as lactose 6-sulfate, sulfated glycosaminoglycans, ascorbic acid 2-sulfate, N-acetylglucosamine 6-sulfate, cellulose polysulfate, etc. are known at present and the sulfatases that hydrolyze the sulfate part of these compounds are collectively referred to as glycosulfatases or carbohydrate sulfatases. The enzyme is present in mollusks, bacteria and molds (Dodgson and Rose, 1975). Habuchi *et al.* (1979) have identified 5 glycosaminoglycan sulfatases from rat skin. They are N-acetylglucosamine and N-acetylgalactosamine 6-sulfate sulfatase, N-acetylgalactosamine 4-sulfate sulfatase, heparan sulfate N-sulfatase and iduronate sulfatase. The deficiency of any one of these may give rise to a specific storage disorder. Iduronate sulfatase has been purified from human liver to 80-fold and shown to be of heterogeneous nature. It had a pH optimum of 4.0 and was free of other sulfatases (Yutaka *et al.*, 1982).

Recently, Benitez and Halver (1982) purified an ascorbic acid 2-sulfatase from rainbow trout. It had a  $M_r$  of 117,500 and a pH optimum of 6.0 and was structurally identical to human arylsulfatase A. Placental N-acetylgalactosamine 6-sulfate sulfatase has been purified by Glossl *et al.* (1979) and shown it to be a glycoprotein of  $M_r$  100,000 by gel filtration. It had a monomeric  $M_r$  of 78,000. A similar enzyme, N-acetylglucosamine 6-sulfate sulfatase has been purified from human urine and shown to be a glycoprotein of  $M_r$  97,000 with a pH optimum of 5.5. It is also active towards glucose 6-sulfate (Basner *et al.*, 1979a). On the other hand, heparan sulfate N-sulfatase, a lysosomal enzyme purified from human urine had a pH optimum of 4.5, pI of 4.7 and a  $M_r$  of 120,000 (Friedman and Arsenis, 1972, 1974).

### *Genetic disorders of sulfate metabolism*

The genetic disorders of sulfate metabolism are characterized by the abnormal accumulation and excretion of different metabolites corresponding to the type of defect (table 2). The accumulation of these compounds have been demonstrated in liver, kidney and brain. Mental retardation is invariably associated with many of these disorders.

#### 3 *Metachromatic leukodystrophy or arylsulfatase A deficiency*

The physiological substrate of arylsulfatase A, cerebroside 3-sulfate accumulates in brain, peripheral nerve, kidney and other visceral organs of the patient as a result of this genetic disorder. It is an autosomal recessive disorder classified into late infantile, juvenile and adult disorders. The first demonstration of arylsulfatase A deficiency in metachromatic leukodystrophy (MLD) came from the work of Austin *et al.* (1963). Diagnosis is carried out by measuring the urinary excretion of cerebroside 3-sulfate, presence of metachromatic deposits in sural nerve and by the deficiency of arylsulfatase A in urine, leukocytes or skin fibroblasts (Brady, 1978). Several studies

Table 2. Storage diseases associated with the deficiency of sulfatases.

Disease	Accumulated product	Enzyme deficient
Metachromatic leukodystrophy	Cerebroside 3-sulfate	Arylsulfatase A (cerebroside 3-sulfatase)
Maroteaux-Lamy syndrome	Dermatan sulfate	Arylsulfatase B (N-acetylgalactosamine 4-sulfatase)
Hunter's syndrome	Dermatan sulfate Heparan sulfate	Iduronate sulfatase
Sanfilippo syndrome A	Heparan sulfate	Heparan N-sulfatase
Morquio's syndrome	Keratan sulfate	N-acetylgalactosamine 6-sulfatase
Placental sulfatase deficiency	Steroid sulfate	Steroid sulfatase
Multiple sulfatase deficiency	Cerebroside 3-sulfate Steroid sulfates Mucopolysaccharides	Arylsulfatase A, B and C, steroid sulfatase, heparan N-sulfatase, iduronate 2-sulfate sulfatase, N-acetylglucosamine 6-sulfate sulfatase, N-acetylgalactosamine 6-sulfate sulfatase
I-Cell disease and pseudo-Hurler polydystrophy	Mucopolysaccharides and glycolipids	Almost all lysosomal enzymes deficient in fibroblasts; enzymes present extracellularly

shown that MLD patients have a mutated sulfatase A, which is immunologically cross-reactive with monospecific antibody, but had very little enzyme activity (Euwelt *et al.*, 1973; Shapira and Nadler, 1975).

There is marked demyelination in the nervous system of patients suffering from MLD. Recent studies have demonstrated pseudoarylsulfatase A deficiency in fibroblasts from healthy individuals (Chang and Davidson, 1983). The enzyme was similar to normal arylsulfatase A except for a slight difference in pI value. Low pseudoarylsulfatase A activity has been reported in leukocytes and skin fibroblasts of healthy members of families having an MLD patient (Dubois *et al.*, 1977; Butterworth *et al.*, 1978). In the late infantile form of MLD, sulfatase A is deficient in brain, liver and spleen (Yamaguchi *et al.*, 1975).

#### *Maroteaux-Lamy syndrome (arylsulfatase B deficiency)*

Arylsulfatase B deficiency is characterized by severe skeletal deformities, gross corneal opacity, hepatosplenomegaly, growth retardation and increased urinary excretion of dermatan sulfate as N-acetylgalactosamine 4-sulfate residues of dermatan sulfate are the physiological substrate of the enzyme. Recent studies (Gasper *et al.*, 1984) have indicated that allogenic bone marrow transplant from a histocompatible donor could reverse an advanced state of arylsulfatase B deficiency in a two year old siamese cat as evidenced by a normalization of urinary glycosaminoglycan excretion, leukocyte arylsulfatase B activity and sustained improvement in clinical symptoms. Hence it would be only a question of time before man can be treated in a similar manner.

#### *Hunter's syndrome (iduronate sulfate sulfatase deficiency)*

This is a sex-linked recessive trait characterized by abnormal accumulation of heparan sulfate, dermatan sulfate and by the deficiency of L-iduronate sulfate sulfatase. It is known to occur only in hemizygous males, while the females who are heterozygous are probably protected from the disorder by an enzyme transfer from normal to abnormal cells. The syndrome can be diagnosed by assaying iduronate sulfate sulfatase activity in serum, lymphocytes, fibroblasts and hair roots (Liebaers and Kaufeld, 1976; Migeon *et al.*, 1977; Yutaka *et al.*, 1978).

#### *Marquio's syndrome*

This syndrome is characterized by abnormal accumulation and excretion of keratan sulfate and chondroitin 6-sulfate and distinguishable from others by such characteristic clinical features as disproportionate dwarfism, spondiloepiphyseal dysplasia, mental anomalies, corneal clouding but normal intellect. Patients are deficient in N-acetylgalactosamine 6-sulfate sulfatase (Singh *et al.*, 1976) and galactose 6-sulfate sulfatase (Yutaka *et al.*, 1982).

#### *Filippo's syndrome type A (heparan sulfate N-sulfatase deficiency)*

physical alterations. It can be diagnosed by assaying heparan sulfate N-sulfatase in fibroblasts and peripheral leukocytes (Schmidt *et al.*, 1977).

### *Placental steroid sulfatase deficiency*

Steroid sulfatase and arylsulfatase C (*p*-nitrophenylsulfatase) are absent in the placenta of pregnant women having this deficiency syndrome. It is characterized by low urinary estrogen excretion compared to normal and pregnant women. This disorder can be easily distinguished from other sulfatase deficiencies both by being sex-linked and causing pregnancy complications (Oakey, 1978). The babies are usually males and develop an ichthyotic skin condition probably caused by excessive accumulation of cholesterol sulfate in the pathological scale (Williams and Elias, 1981) in the first year. Corneal dystrophy has been described in patients with X-linked ichthyosis and some female carriers (Sever *et al.*, 1968).

Recent studies have shown that steroid sulfatase deficient placental microsomes have a significantly lower percentage of phosphatidylcholine as compared to normal placental microsomes (McKee and France, 1983). This finding is important especially since phosphatidylcholine can stimulate steroid sulfatase (McNaught and France, 1980). The disorder can be diagnosed by subjecting the patients to dehydroepiandrosterone and dehydroepiandrosterone sulfate loading tests (Braunstein *et al.*, 1976) or by measuring the cholesterol sulfate content in the pathological scales (Williams and Elias, 1981).

### *Multiple sulfatase deficiency*

It is an autosomal recessive trait characterized by a profound deficiency of arylsulfatase C along with arylsulfatase A, B and steroid sulfatase resulting in the accumulation of cerebroside 3-sulfate, steroid sulfates and mucopolysaccharides (Eto *et al.*, 1974; Murphy *et al.*, 1971; Austin, 1973). The suggestion that the deficient arylsulfatases must be having a common subunit controlled by a single gene (Moser *et al.*, 1972) has been ruled out by the fact that arylsulfatase A and B are assigned to different chromosomes (DeLuca *et al.*, 1979; Hors-Cayla *et al.*, 1979). Nine different sulfatases (arylsulfatase A, B and C, cholesterol sulfatase, dehydroepiandrosterone sulfatase, iduronate 2-sulfate sulfatase, N-acetylgalactosamine 6-sulfatase, N-acetylglucosamine 6-sulfate sulfatase and heparan sulfate N-sulfatase) are shown to be deficient in this syndrome (Banser *et al.*, 1979b). Studies by Chang *et al.* (1983) using cultured fibroblasts from 7 different patients have suggested typing of the patients into 3 groups: (i) deficient in A, B and C; (ii) deficient in A and C with half or near normal levels of B and (iii) same as in (ii), but arylsulfatase A activity bands are detectable.

Recent studies using fibroblasts from multiple sulfatase deficient patients demonstrate a decreased stability of arylsulfatase A and the 47,000 dalton form of arylsulfatase B (Steckel *et al.*, 1985) and steroid sulfatase (Horwitz *et al.*, 1986). The root cause of this disorder is still not known. They are probably secondary manifestations

## References

- Agogbua, S. I. O. and Wynn, C. H. (1976) *Biochem. J.*, **153**, 415.
- Austin, J. H., Balasubramanian, A. S., Pattabiraman, T. N., Saraswathi, S., Basu, D. K. and Bachhawat, B. K. (1963) *J. Neurochem.*, **10**, 805.
- Austin, J. H. (1973) *Arch. Neurol.*, **28**, 258.
- Balasubramanian, A. S. and Bachhawat, B. K. (1964) *J. Neurochem.*, **11**, 877.
- Balasubramanian, A. S. and Bachhawat, B. K. (1965) *Biochim. Biophys. Acta*, **106**, 218.
- Balasubramanian, A. S. and Bachhawat, B. K. (1970) *Brain Res.*, **20**, 341.
- Balasubramanian, A. S. (1976) *Indian J. Biochem. Biophys.*, **13**, 325.
- Balasubramanian, K. A. and Bachhawat, B. K. (1975) *Biochim. Biophys. Acta*, **403**, 113.
- Banerjee, R. K. and Roy, A. B. (1966) *Mol. Pharmacol.*, **2**, 56.
- Basner, R., Kresse, H. and von Figura, K. (1979a) *J. Biol. Chem.*, **254**, 1151.
- Basner, R., von Figura, K., Glossl, J., Klein, U., Kresse, H. and Mlekusch, W. (1979b) *Pediatr. Res.*, **13**, 1316.
- Benitez, L. V. and Halver, J. E. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5445.
- Bleau, G., Lalumiere, G., Chapdelaine, A. and Roberts, K. D. (1975) *Biochim. Biophys. Acta*, **375**, 220.
- Bleszynski, W. S., Leznicki, A. and Lewosz, J. (1969) *Enzymology*, **37**, 314.
- Bostrom, H. and Wengle, B. (1967) *Acta. Endocrinol.*, **56**, 691.
- Brady, R. O. (1978) *Annu. Rev. Biochem.*, **47**, 687.
- Braunstein, G. D., Zeil, F. H., Allen, A., Van de Velde, R. and Wade, M. E. (1976) *Am. J. Obstet. Gynecol.*, **126**, 716.
- Burns, G. R. J. (1983) *Biochim. Biophys. Acta*, **759**, 199.
- Butterworth, J., Broadhead, D. M. and Keay, A. J. (1978) *Clin. Genet.*, **14**, 213.
- Chang, P. L. and Davidson, R. G. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7323.
- Chang, P. L., Rosa, N. E., Ballantyne, S. R. and Davidson, R. G. (1983) *J. Int. Metab. Dis.*, **6**, 167.
- Chen, L. J. and Thaler, M. M. (1984) *Hepatology*, **4**, 1195.
- Chen, L. J. and Segel, I. H. (1985) *Arch. Biochem. Biophys.*, **24**, 371.
- Cumar, F. A., Barr, H. S., Maecioni, H. and Cuputto, R. (1968) *J. Biol. Chem.*, **243**, 3807.
- Crocker, M. and Craig, I. (1983) *Nature (London)*, **303**, 721.
- Das, P. K. and Bishayee, S. (1980) *FEBS Lett.*, **111**, 43.
- DeLuca, S., Richmond, M. E. and Silbert, J. E. (1973) *Biochemistry*, **12**, 3911.
- DeLuca, C., Brown, J. A. and Shows, T. B. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1957.
- Delfert, D. M. and Conrad, H. E. (1985) *J. Biol. Chem.*, **260**, 14446.
- Dietrich, C. P. (1984) *Braz. J. Med. Biol. Res.*, **17**, 5.
- Dodgson, K. S., Spencer, B. and Thomas, J. (1954) *Biochem. J.*, **56**, 177.
- Dodgson, K. S. and Rose, F. A. (1970) in *Metabolic conjugation and metabolic hydrolysis* (ed. W. H. Fishman) (New York: Academic Press) vol. 1, p. 239.
- Dodgson, K. S. and Rose, F. A. (1975) in *Metabolic Pathways* (ed. D. M. Greenberg) (New York: Academic Press) vol. 7, p. 359.
- Dodgson, K. S., Spencer, B. and Wynn, C. H. (1956) *Biochem. J.*, **62**, 500.
- Dolly, J. O., Dodgson, K. S. and Rose, F. A. (1972) *Biochem. J.*, **128**, 337.
- Dubois, G., Harzer, K. and Bauman, N. (1977) *Am. J. Hum. Genet.*, **29**, 191.
- Eto, Y., Rampini, S., Wiesmann, U. and Hershkowitz, N. N. (1974) *J. Neurochem.*, **23**, 1161.
- Farooqui, A. A. and Bachhawat, B. K. (1972) *Biochem. J.*, **126**, 1025.
- Farooqui, A. A. and Roy, A. B. (1976) *Biochem. Biophys. Acta*, **452**, 431.
- Farooqui, A. A. (1976) *Experientia*, **32**, 1242.
- Farooqui, A. A. (1980) *Clin. Chim. Acta*, **100**, 285.
- Farooqui, A. A. (1981) *Adv. Lipid Res.*, **18**, 159.
- Fitzgerald, J. W. (1973) *Biochem. J.*, **136**, 361.
- Fleischer, B. and Smigel, M. (1978) *J. Biol. Chem.*, **253**, 1725.
- Fluharty, A. L., Stevens, R. L., Fung, D., Peak, S. and Kihara, H. (1975) *Biochem. Biophys. Res. Commun.*, **64**, 955.
- French, A. P. and Warren, J. C. (1967) *Biochem. J.*, **105**, 233.
- Friedman, Y. and Arsenis, C. (1972) *Biochem. Biophys. Res. Commun.*, **48**, 1133.



- Gasa, S., Makita, A., Kameya, T., Kodama, T., Koide, T., Tsumuraya, M. and Komai, T. (1981) *Eur. J. Biochem.*, **116**, 497.
- Gasa, S. and Makita, A. (1983) *J. Biol. Chem.*, **258**, 5034.
- Gaspar, P. W., Thrall, M. A., Wenger, D. A., Macy, D. W., Ham, L., Dornsife, K., McBiles, K., Quackenbush, S. L., Kesel, M. L., Gillette, E. L. and Hoover, E. A. (1984) *Nature (London)*, **312**, 467.
- Gniot-Szulszycka, J. (1972) *Acta Biochim. Pol.*, **19**, 181.
- Glossl, J., Truppe, W. and Kresse, H. (1979) *Biochem. J.*, **181**, 37.
- Gregory, J. D. and Robbins, P. W. (1960) *Annu. Rev. Biochem.*, **29**, 347.
- Habuchi, H., Tsuji, M., Nakanishi, Y. and Suzuki, S. (1979) *J. Biol. Chem.*, **254**, 7570.
- Habuchi, O. and Miyashita, N. (1982) *Biochim. Biophys. Acta*, **717**, 414.
- Harris, B. W., Daniel, W. L. and Abbas, K. J. (1982) *Experientia*, **38**, 73.
- Hart, R. F., Renskers, K. J., Nelson, E. B. and Roth, J. A. (1979) *Life Sci.*, **24**, 125.
- Harzer, K., Stinshoff, K., Mraz, W. and Jatzkewitz, H. (1973) *J. Neurochem.*, **20**, 279.
- Hasegawa, E., Delbruck, A. and Lipman, F. (1961) *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **20**, 86.
- Hess, G. (1983) *Eur. J. Biochem.*, **135**, 505.
- Hille, A., Rosa, P. and Huttner, W. B. (1984) *FEBS Lett.*, **177**, 129.
- Hook, M., Kjellén, L., Johansson, S. and Robinson, J. (1984) *Annu. Rev. Biochem.*, **53**, 847.
- Hors-Cayla, M. C., Heuertz, S., Van Cong, N., Weil, D. and Frezal, J. (1979) *Hum. Genet.*, **49**, 33.
- Horwitz, A. L., Warshawsky, L., King, J. and Burns, G. (1986) *Biochem. Biophys. Res. Commun.*, **135**, 389.
- Huttner, W. B. (1982) *Nature (London)*, **299**, 273.
- Iwamori, M., Moser, H. W. and Kishimoto, Y. (1976a) *J. Neurochem.*, **27**, 1389.
- Iwamori, M., Moser, H. W. and Kishimoto, Y. (1976b) *Arch. Biochem. Biophys.*, **174**, 199.
- James, G. T. (1979) *Arch. Biochem. Biophys.*, **197**, 57.
- James, G. T. and Austin, J. H. (1979) *Clin. Chim. Acta*, **98**, 103.
- Kaji, A. and Gregory, J. D. (1959) *J. Biol. Chem.*, **234**, 3007.
- Kottgen, E., Reutter, W. and Gerok, W. (1978) *Eur. J. Biochem.*, **82**, 279.
- Kirkpatrick, R. B., Wildermann, N. M. and Killenberg, P. G. (1985) *Am. J. Physiol.*, **248**, G639.
- Kureha, Y. and Eto, Y. (1983) *J. Biochem.*, **94**, 1489.
- Laidler, P. M., Waheed, A. and Van Etten, R. L. (1985) *Biochim. Biophys. Acta*, **827**, 73.
- Lakshmi, S. and Balasubramanian, A. S. (1979) *Biochim. Biophys. Acta*, **567**, 184.
- Lakshmi, S. and Balasubramanian, A. S. (1984) *J. Biosci.*, **6**, 79.
- Langlais, J., Zollinger, M., Plante, L., Chapdelaine, A., Bleau, G. and Roberts, K. D. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7266.
- Lee, R. W. H. and Huttner, W. B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6143.
- Liebaers, I. and Neufeld, E. F. (1976) *Pediatr. Res.*, **10**, 733.
- Lin, Y. N. and Horowitz, M. I. (1980) *Steroids*, **36**, 697.
- Lingwood, C. A. (1985) *Biochem. J.*, **231**, 393.
- Littlewood, J., Glover, V. and Sandler, M. (1982) *Lancet.*, **1**, 983.
- Liu, Ming-Cheh. and Lipmann, F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3695.
- Lugg, J. W. H. (1945) *Annu. Rev. Biochem.*, **14**, 263.
- Marcus, C. J., Sekura, R. D. and Jakoby, W. B. (1980) *Anal. Biochem.*, **107**, 296.
- Mathew, J. and Balasubramanian, A. S. (1982) *J. Neurochem.*, **39**, 1205.
- Mathew, J. and Balasubramanian, A. S. (1984) *Dev. Neurosci.*, **6**, 278.
- McGovern, M. M., Vine, D. T., Haskins, M. E. and Desnick, R. J. (1982) *J. Biol. Chem.*, **257**, 12605.
- McKee, J. W. A. and France, J. T. (1983) *J. Steroid Biochem.*, **18**, 309.
- McKhann, G. M., Levy, R. and Ho, W. (1965) *Biochem. Biophys. Res. Commun.*, **20**, 109.
- McNaught, R. W. and France, J. T. (1980) *J. Steroid Biochem.*, **13**, 363.
- Migeon, B. R., Sprenkle, J. A., Liebaers, I., Scott, J. F. and Neufeld, E. F. (1977) *Am. J. Hum. Genet.*, **29**, 448.
- Milewich, L., Garcia, R. L. and Gerrity, L. W. (1984) *J. Steroid Biochem.*, **21**, 529.
- Milewich, L. and Garcia, R. L. (1985) *J. Clin. Endocrinol. Metab.*, **61**, 812.
- Mitsushashi, K., Maru, A., Koyanagi, T., Ishibashi, T., Imai, Y., Gasa, S., Taniguchi, N. and Makita, A. (1984) *Jpn. J. Exp. Med.*, **54**, 211.
- Moriyasu, M., Ito, A. and Omura, T. (1982) *J. Biochem.*, **92**, 1189.
- Moser, H. W., Sujita, M., Harbison, M. D. and Williams, M. (1972) in *Sphingolipids, sphingolipidoses and*

- and H. J. Gross) (New York: Academic Press) p. 67.
- Nakamura, M., Gasa, S. and Makita, A. (1984) *J. Biochem.*, **96**, 207.
- Neuwelt, E., Kohler, P. F. and Austin, J. (1973) *Immunochimistry*, **10**, 767.
- Niinobe, M., Tamura, Y., Arima, T. and Fujii, S. (1979) *Cancer Res.*, **39**, 4212.
- Nissen, P. and Benson, A. A. (1961) *Science*, **134**, 1759.
- Noel, H., Plante, L., Bleau, G., Chapdelaine, A. and Roberts, K. D. (1983) *J. Steroid Biochem.*, **19**, 1591.
- Oakey, R. E. (1978) *Clin. Endocrinol.*, **9**, 81.
- Otten, M. H., Mol, J. A. and Visser, T. J. (1983) *Nature (London)*, **221**, 81.
- Parsons, T. F. and Pierce, J. G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7089.
- Prosser, C. I. and Roy, A. B. (1980) *Biochim. Biophys. Acta*, **613**, 130.
- Prost, O., Nicollier, M., Laurent, R. and Adessi, G. L. (1985) *Arch. Dermatol. Res.*, **277**, 195.
- Rein, G., Glover, V. and Sandler, M. (1982) *Biochem. Pharmacol.*, **31**, 1893.
- Rein, G., Glover, V. and Sandler, M. (1984) *J. Neurochem.*, **42**, 80.
- Richmond, M. E., DeLuca, S. and Silbert, J. E. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 263.
- Richmond, M. E., DeLuca, S. and Silbert, J. E. (1973) *Biochemistry*, **12**, 3898, 3904.
- Ropers, H. H. and Wieberg, U. (1982) *Nature (London)*, **296**, 766.
- Roy, A. B. (1960a) *Adv. Enzymol.*, **22**, 205.
- Roy, A. B. (1960b) *Biochem. J.*, **74**, 49.
- Roy, A. B. (1985) *Biochim. Biophys. Acta*, **827**, 84.
- Rueter, E. R. and Kresse, H. (1984) *J. Biol. Chem.*, **259**, 11771.
- Sarafian, T. A., Fluharty, A. L. and Kihara, H. (1984) *Biochem. Med.*, **31**, 80.
- Schmidt, R., von Figura, K., Paschke, E. and Kresse, H. (1977) *Clin. Chim. Acta*, **80**, 7.
- Sever, R. J., Frost, P. and Weinstein, G. (1968) *J. Am. Med. Assoc.*, **206**, 2283.
- Shapira, E. and Nadler, H. L. (1975) *J. Pediatr.*, **86**, 881.
- Singh, J., DiFerrante, N., Niebes, P. and Tavella, D. (1976) *J. Clin. Invest.*, **57**, 1036.
- Singer, S. S., Lee, A. M. A. and Kutzer, T. (1980) *Biochem. Pharmacol.*, **29**, 3181.
- Simard, J. P. S., Ameen, M. and Chang, P. L. (1985) *Biochem. Biophys. Res. Commun.*, **128**, 1388.
- Sorkin, B. C., Hoffman, S., Edelman, G. M. and Cunningham, B. A. (1984) *Science*, **225**, 1476.
- Spencer, B. (1960) *Biochem. J.*, **77**, 294.
- Steckel, F., Hasilik, A. and von Figura, K. (1983) *J. Biol. Chem.*, **258**, 14322.
- Steckel, F., Hasilik, A. and von Figura, K. (1985) *Eur. J. Biochem.*, **151**, 141-147.
- Stevens, R. L., Fluharty, A. L., Killgrove, A. R. and Kihara, H. (1977) *Biochim. Biophys. Acta*, **481**, 549.
- Stone, G. C., Hammerschlag, R. and Bobinski, J. A. (1984) *Cell Mol. Neurobiol.*, **4**, 249.
- Suzuki, S., Trenn, R. H. and Strominger, J. L. (1961) *Biochim. Biophys. Acta*, **50**, 169.
- Tennekoon, G., Aitchison, S. and Zaruba, M. (1985) *Arch. Biochem. Biophys.*, **240**, 932.
- Tseng, L., Lee, L. Y. and Mazella, J. (1985) *J. Steroid Biochem.*, **22**, 611.
- Uehara, Y., Gasa, S., Makita, A., Sakurada, K. and Miyazaki, T. (1983) *Cancer Res.*, **43**, 5618.
- Unsworth, C. D., Hughes, J. and Morley, J. S. (1982) *Nature (London)*, **295**, 519.
- Vargas, F., Frerot, O., Dan, T. T. M. and Schwartz, J. C. (1985) *Biochemistry*, **24**, 5938.
- Vestermark, A. and Bostrom, H. (1959) *Exp. Cell Res.*, **18**, 174.
- Waheed, A. and Van Etten, R. L. (1980) *Arch. Biochem. Biophys.*, **203**, 11.
- Waheed, A., Steckel, F., Hasilik, A. and von Figura, K. (1983) *Am. J. Hum. Genet.*, **35**, 228.
- Waheed, A. and Van Etten, R. L. (1985a) *Int. J. Peptide Protein Res.*, **26**, 362.
- Waheed, A. and Van Etten, R. L. (1985b) *Biochim. Biophys. Acta*, **847**, 53.
- Weller, P. F. and Austen, K. F. (1983) *J. Clin. Invest.*, **71**, 114.
- Whittemore, R. M., Pearce, L. B., Roth, J. A. (1985) *Biochemistry*, **24**, 2477.
- Wieland, F. (1982) *Trends Biochem. Sci.*, **7**, 308.
- Williams, M. L. and Elias, P. M. (1981) *J. Clin. Invest.*, **68**, 1404.
- Yamaguchi, S., Aoki, K., Handa, S. and Yamukawa, T. (1975) *J. Neurochem.*, **24**, 1087.
- Young, R. W. (1973) *J. Cell Biol.*, **57**, 175.
- Young, W. F. Jr., Laws, E. R. Jr., Sharbrough, F. W. and Weinshilboum, R. M. (1985) *J. Neurochem.*, **44**, 1131.
- Yu, P. H., Rozdilsky, B. and Boulton, A. A. (1985) *J. Neurochem.*, **45**, 836.
- Yutaka, T., Fluharty, A. L., Stevens, R. L. and Kihara, H. (1978) *Am. J. Hum. Genet.*, **30**, 575.
- Yutaka, T., Okada, S., Kato, T., Inui, K. and Yabuuchi, H. (1982) *Clin. Chim. Acta*, **122**, 169.



# Metabolism of an isolated brain perfused with perfluoro blood substitute

B. MUKHERJI and H. A. SLOVITER\*

Harrison Department of Surgical Research and Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

**Abstract.** An unanesthetized, isolated, perfused rat brain, consisting of the skull and its contents with nearly all other tissues removed, has metabolic and electrical activity similar to that of the brain of the intact rat with its blood-brain barrier intact. Its use yielded results that are difficult or impossible to obtain from *in vitro* preparations or *in vivo*. With the perfused brain it was shown that, mannose can completely replace glucose as metabolic substrate, that insulin has no direct effect on the brain, in the absence of added substrate glutamate is metabolized to aspartate, the brain does not metabolize ethanol, and morphine probably inhibits mitochondrial oxidative activity. Since the use of a perfluoro blood substitute to perfuse the brain avoids the optical interference caused by haemoglobin, it was possible to measure changes in the oxidation-reduction state of NADH by surface fluorometry of the cerebral cortex.

**Keywords.** Brain metabolism; perfused brain; perfluoro chemicals; blood substitute.

## Use of an isolated brain

When an animal is treated with a drug or other substance that causes some change in the function of the brain, the observed change may be due to any or all of 3 following actions: (i) a direct action of the substance on the brain, (ii) an action on the brain of a metabolite of the substance, and (iii) an effect on the brain which is secondary to the action of the substance or its metabolite on another organ. It is usually not possible to determine which of these actions is responsible for the observed effect. Further difficulties in the interpretation of *in vivo* experiments result from the artifacts due to the excitement or other stimuli caused by handling of the animal or by administration of a drug, or due to the use of an anesthetic agent.

Studies *in vitro* with brain tissue preparations (slices, homogenates, subcellular particles, etc.) provide useful information about the direct action of agents on tissue. These *in vitro* methods often permit the separation of the effects of a drug from those of its metabolites; however such experiments indicate those activities that may occur, but are not necessarily those that do occur, under physiological conditions. It is questionable whether measurements made with isolated pieces of brain tissue having no blood circulation, no blood-brain barrier, and sometimes little or no neural organization can be valid indicators of the functions of the intact brain. Furthermore, these tissue preparations usually contain injured cells and sometimes are incubated in an artificial and nonphysiological environment.

Many of the limitations and disadvantages of the *in vivo* and *in vitro* methods can be avoided by the use of an isolated brain maintained in viable condition by perfusion

---

\*To whom all correspondence should be addressed.

Abbreviations used: EEG, Electroencephalogram; GABA,  $\gamma$ -aminobutyric acid; DMSO, dimethyl

from other tissues in varying degrees in different preparations. Various degrees of vascular isolation, necessary to avoid the influence of other organs, have been achieved in different preparations. Neural isolation has ranged from complete isolation, with removal of both cranial and spinal nerve input, to no isolation at all in different perfused preparations. In general, the perfused brain preparations resembled the brain of the intact animal in both its metabolic and electrical activities. However, the perfused brain preparation is not identical to the brain of the intact animal.

The perfused brain preparation permits control of variables, such as blood flow, temperature, and the composition of circulating blood, that are difficult or impossible to control in the intact animal. For example, it was possible to study changes in an isolated brain when neither glucose nor any other metabolic substrate was present in the perfusion fluid (Mukherji *et al.*, 1971) whereas a blood glucose level of zero in the intact animal would rapidly have resulted in convulsions and death. Studies with radioactive isotopes are greatly facilitated by the isolated brain preparation, since the small volume of perfusion fluid and the absence of competition from other organs can markedly reduce the amount of radioactivity required for tracer studies.

### Perfused brain preparations

The first perfused brain preparation was that of Heymans and Kochmann (1904) in which the decapitated head of a dog was perfused through its carotid arteries by anastomosis to the carotid arteries of a second dog. In a later modification of the preparation by Bouckaert and Jourdan (1956), the brain was perfused by a pump.

Another early perfused brain preparation was that of Schmidt (1928), in which the arterial circulation to the brain of a dog or cat was partially occluded and then perfusion fluid (blood) was pumped into the vertebral artery at a pressure higher than that in the aorta. This principle of circulatory isolation of the brain was also used by Moss (1964), to perfuse a calf brain *via* a carotid artery, and by Thompson *et al.* (1968), to perfuse the head of a rat *via* the aorta.

Geiger and Magnes (1947) developed a perfused cat brain preparation in which the arterial circulation to the brain was not isolated, but the venous outlets from the brain were occluded and a single venous outlet from a cerebral venous sinus was made. The central and peripheral nervous systems were kept intact, so they were able to observe a variety of neurological functions during their experiments.

White *et al.* (1964) prepared the most completely isolated of the perfused brain preparations when they removed all tissues except a small piece of the skull from the brain of a monkey. Circulatory isolation was complete, and neural isolation was almost complete, since all cranial (except the eighth) and spinal nervous connections were severed. They also used a preparation in which the isolated monkey brain was kept inside the skull and concluded that retention of the skull was desirable for protection and maintenance of the environment of the brain. Presumably, removal of the skull and dura was conducive to the development of cerebral edema.

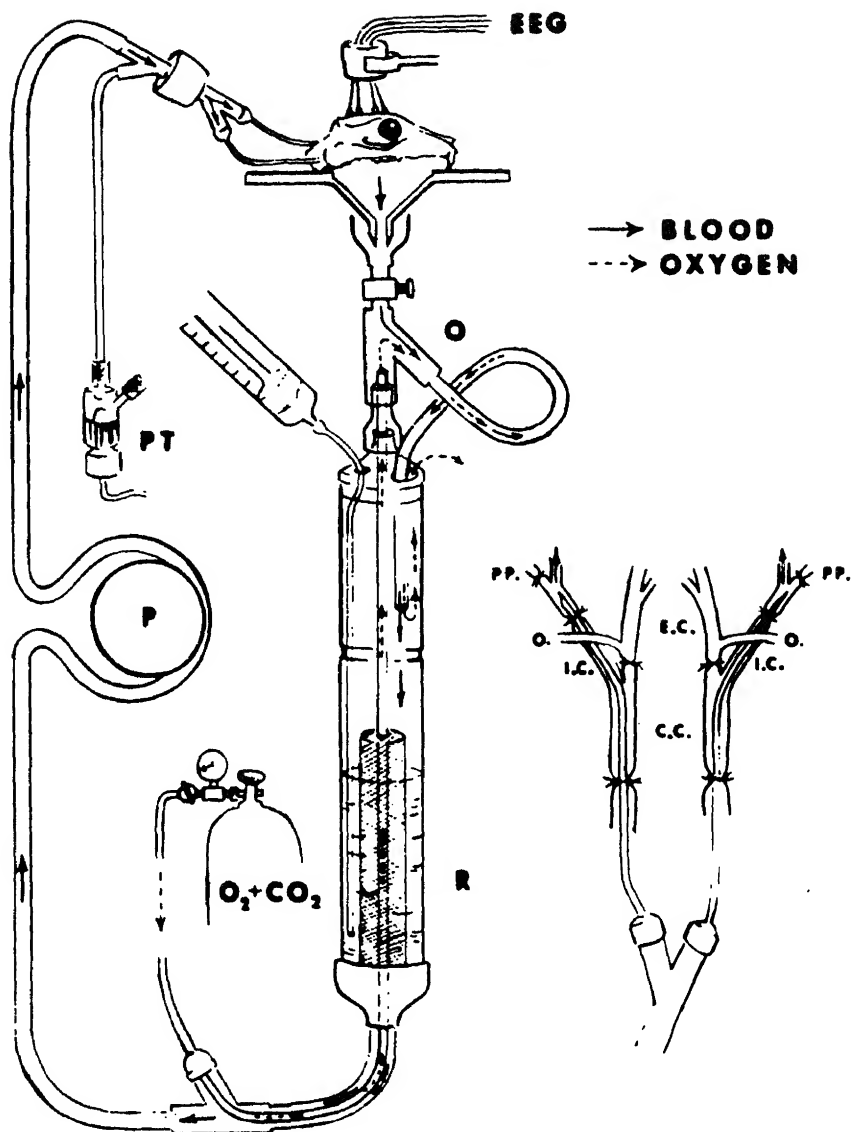
We have used the isolated rat brain preparation developed by Andjus *et al.* (1967), which consisted of the skull and its contents with nearly all extracranial tissue removed. In contrast to all the other perfused brain preparations, this preparation

that was adequately anesthetized for the surgical procedure by deep hypothermia (rectal temperature 16°C). The use of a chemical anesthetic agent is undesirable because it is known (Smith and Wollman, 1972) that the anesthetic drugs have marked effects on the metabolic activities of the brain. Even short-acting or volatile anesthetic agents may not be entirely eliminated from an isolated brain which has no liver or kidney associated with it, and the effects of the agent may persist for a considerable time after the agent is removed. In addition, the use of hypothermia during surgery diminishes bleeding, simplifies the surgical procedure, and reduces the hazard of the occurrence of shock. Furthermore, the decreased metabolic requirements of the brain during hypothermia reduce the hazard of damage to the brain by transient anoxia.

The isolated rat brain was perfused through catheters placed in the internal carotid arteries, with all other arterial channels occluded. The position of the catheters and the arrangement of the blood vessels are illustrated schematically in figure 1. Perfusion at a slow rate was started before the vertebral arteries were ligated so that at no time was there an interruption of circulation through the brain. As the extracranial tissues were removed, the perfusion rate was gradually increased. The vertebral column and spinal cord were cut and the completed preparation was mounted above the collecting funnel of the perfusion system as shown in figure 1. All venous channels were open and the venous drainage ran by gravity into the collecting funnel, then into the oxygenator-reservoir, from which it was pumped back to the brain. The entire surgical procedure was performed by a single operator without assistance in less than 1 h.

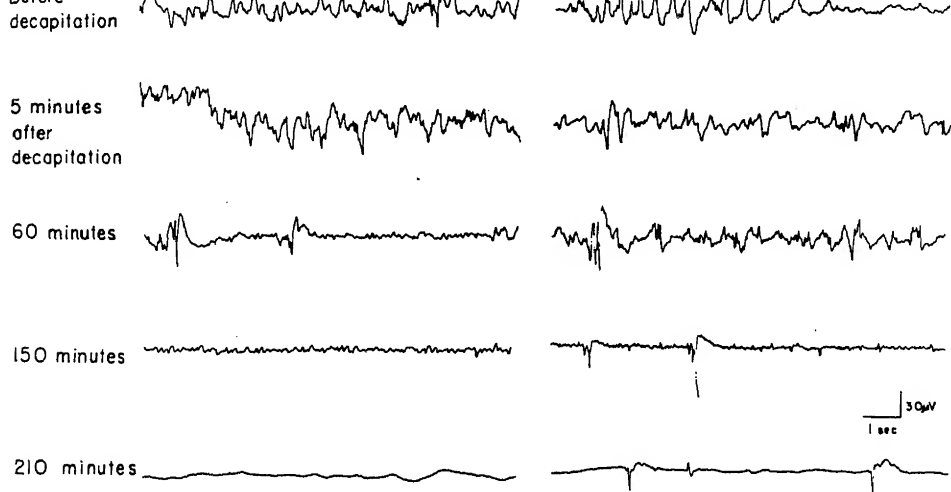
### **Perfusion with perfluoro blood substitute**

The perfusion fluid used in our earlier experiments was a freshly prepared suspension (25% hematocrit) of washed dog erythrocytes in an 8% solution of purified bovine serum albumin in Krebs-Ringer bicarbonate buffer containing 11 mM glucose. In later experiments, Sloviter and Kamimoto (1967) found that the erythrocytes could be replaced advantageously by ultrasonically dispersed particles (*ca.* 0.2  $\mu$ m diam) of fluorochemical which transported oxygen and carbon dioxide in the physically dissolved form. The fluorochemical was a commercial perfluoro compound (perfluorobutyltetrahydrofuran), which is a colorless, inert liquid. When equilibrated with oxygen, it contains about 10% more oxygen than does an equal volume of erythrocytes. A 15% (v/v) emulsion of this perfluoro compound in the same solution of bovine albumin that was used with erythrocytes was an entirely satisfactory perfusion fluid in maintaining the activities of the isolated brain. The spontaneous electrical activity of the isolated brain (electroencephalogram, EEG) is a very sensitive criterion of its functional condition. Cessation of perfusion for 30 s caused loss of the EEG activity, and when the isolated brain was perfused with bovine albumin solution alone, the EEG never persisted for more than 5 min. Figure 2 shows EEG recordings of an isolated brain perfused with the suspension of erythrocytes and those of another brain perfused with the emulsified perfluoro compound. The nature and duration of the EEG activity of the brain perfused with perfluoro compound were very similar to those of the brain perfused with erythrocytes. This indicates that the



**Figure 1.** Schematic diagram of the system for perfusion of the isolated rat brain preparation. Venous efflux from the preparation is dispersed by the stream of gas (5% CO<sub>2</sub>/95% O<sub>2</sub>) into short segments and carried through the side branch of the oxygenator (O) into the reservoir (R). The roller pump (P) has a continuously variable speed. Perfusion pressure is monitored by a pressure transducer (PT). Reproduced with permission from (Andjus *et al.*, 1967).

perfluoro compound is adequately performing the gas transport functions of the



**Figure 2.** Spontaneous electroencephalographic activity of an isolated rat brain perfused with a suspension of erythrocytes (recordings on the left) and another perfused with a suspension of the dispersed fluorochemical FX-80 (recordings on the right) at various times. Reproduced with permission from Sloviter and Kamimoto (1967).

### Blood-brain barrier in perfused brain

In the isolated, perfused rat brain preparation, the blood-brain barrier appears to be intact. This is indicated by the fact that pyruvate cannot replace glucose as a substrate for the maintenance of the electrical and metabolic activities of the brain. It is known (Elliott *et al.*, 1942) that brain slices or homogenates utilize pyruvate readily and that when both glucose and pyruvate are available, pyruvate is utilized preferentially by brain tissue *in vitro*. However, the brain of the intact animal is apparently unable to utilize pyruvate in the circulating blood at an appreciable rate (Maddock *et al.*, 1939). A further indication that the blood-brain barrier was intact in the isolated, perfused rat brain was the fact that the free amino acids of the brain tissue did not leak out during perfusion. After 2 h of perfusion of the isolated rat brain, no detectable amounts of glutamate, aspartate, N-acetylaspartate, or  $\gamma$ -aminobutyric acid (GABA), and only a trace of glutamine were present in the perfusion fluid (Andjus *et al.*, 1967). Furthermore, the addition of glutamate to the perfusion fluid did not cause an increase in the concentration of glutamate in the brain tissue. Therefore, it appears from these measurements that the blood-brain barrier functions in the perfused rat brain in the same way as in the brain of the intact rat.

### Metabolic studies

The first dependable measurement of the rate of oxygen consumption by the intact brain was obtained by Chute and Smith (1939) with a perfused cat brain preparation.



animal is the difficulty in obtaining reliable measurements of the rate of cerebral flow. This difficulty is avoided in the perfused brain because the rate of blood flow is easily controlled. Thus, these authors perfused the cat brain at 60–90 ml/min and observed arteriovenous differences in oxygen concentrations which gave values for oxygen consumed by the brain of 3.3–5.0 ml O<sub>2</sub>/min per 100 g.

Geiger and Magnes (1947) and Geiger (1958) made a large number of studies on the metabolism of the perfused cat brain. Sacks (1961) evaluated the metabolic state of the brain obtained with this perfused cat brain preparation.

Our isolated rat brain preparation utilized glucose at an approximately linear rate for more than 3 h when perfused in a closed circuit with a small volume of perfusion fluid. From measurements of changes in glucose concentration and the known flow rate of the perfusion fluid, the rate of utilization of glucose by the brain was calculated. In experiments performed at 37°C, Sloviter and Yamada (1971) obtained values for glucose consumption by the perfused rat brain of 35  $\mu$ mol/h/g. In the intact rat, Cremer (1970) obtained a value for glucose consumption by brain of 32  $\mu$ mol/h/g. Lactate accumulated in the fluid perfusing the isolated rat brain at about 10  $\mu$ mol/h/g. A considerable proportion of this lactate is produced by injured tissue (the spinal cord where it is ligated and cut) and by bone and remnants of muscle. In our experiments the contribution of extracranial and injured tissue was excluded by establishing a single channel of venous return from a catheter placed in the jugular venous sinus, and the observed rate of lactate production by the perfused brain was much lower.

The intermediary metabolism of the perfused rat brain has been characterized by measurements of the concentrations of the intermediates of glycolysis in the perfused brain tissue (Ghosh *et al.*, 1976). These values and also the concentrations of inorganic phosphate and the adenine nucleotides are listed in table 1. The effects of different agents or conditions on these values will give some indication of the nature of the alterations in metabolism. Changes in the levels of the glycolytic intermediates will indicate an effect on the regulation of glycolysis, while changes in the levels of the energy reserves will suggest an effect on mitochondrial function.

The isolated, perfused rat brain has also been used to study the ability of the brain to utilize substrates other than glucose. It was generally believed that the mammalian brain has an absolute requirement for glucose and that no other substrate can entirely replace it. Experiments (Ghosh *et al.*, 1972) with the isolated, perfused rat brain have shown that its electrical and metabolic activity were maintained as well with mannose as the metabolic substrate as with glucose. The glycolytic intermediates, the energy reserves, and the oxidative status of the brain tissue perfused with mannose were not materially different from those of the tissue perfused with glucose.

## Amino acids

The concentrations of the free amino acids in the perfused brain tissue were measured (table 2). When the rat brain was perfused with fluid containing no glucose or other metabolic substrate, there were marked reductions in the levels of glycine, aspartic acid and glutamine and an increase in the aspartic acid level, almost equal to

energy reserves in cerebral tissue of isolated, perfused rat brains.

	nmol/g wet wt $\pm$ SD
Glycogen (as glucose)	860 $\pm$ 100
Glucose-6-phosphate	73.0 $\pm$ 8.4
Fructose-6-phosphate	20.0 $\pm$ 4.0
Mannose-6-phosphate	228 $\pm$ 36
Fructose-1,6-diphosphate	93 $\pm$ 11
Triose phosphate	41.1 $\pm$ 4.6
3-Phosphoglycerate	39.8 $\pm$ 5.2
2-Phosphoglycerate	14.8 $\pm$ 1.9
Phosphoenolpyruvate	7.7 $\pm$ 1.2
Pyruvate	60.8 $\pm$ 6.4
Lactate	1,850 $\pm$ 400
Creatine phosphate	2,550 $\pm$ 230
ATP	1,990 $\pm$ 140
ADP	720 $\pm$ 50
AMP	240 $\pm$ 40
ATP + ADP + AMP	2,950 $\pm$ 130
Lactate/pyruvate	31 $\pm$ 7
ATP/ADP	2.93 $\pm$ 0.37

**Table 2.** Concentration of free amino acids in cerebral tissue of isolated, perfused rat brains.

	$\mu$ mol/g wet wt $\pm$ SD
Glutamic acid	6.15 $\pm$ 0.56
Glutamine	7.39 $\pm$ 0.31
GABA	2.36 $\pm$ 0.08
Aspartic acid	2.24 $\pm$ 0.12
N-Acetylaspartic acid	6.94 $\pm$ 0.31

of the decreases in glutamic acid and glutamine. These results suggested that glutamate and glutamine had been converted to ketoglutarate, which was then oxidized *via* the tricarboxylic acid cycle to oxaloacetic acid. Since acetyl-coenzyme A was not available (owing to the lack of glucose) to react with the oxaloacetate, it was converted to aspartic acid. This partial oxidation of glutamate and glutamine to aspartate provided some energy for the brain, which was deprived of its normal source of metabolic energy.

The presence of a large amount of N-acetylaspartic acid (Tallan *et al.*, 1954) in the brain and its virtual absence from other tissue suggests that this free amino acid has some specialized function in the brain. However, no function has been found. When the isolated rat brain was perfused with fluid containing radioactive acetate (Mukherji and Sloviter, 1973), much of the radioactivity was incorporated into glutamate, glutamine, GABA, and aspartate, but very little into N-acetylaspartic. These results indicated that the N-acetylaspartic acid was metabolically inert and that it was not

## Ethanol

There are conflicting reports concerning the presence of alcohol dehydrogenase in mammalian brain and the ability of the brain to metabolize ethanol. Most of the early studies with brain tissue preparations *in vitro* concluded that brain tissues do not metabolize ethanol (Beer and Quastel, 1958). The question was opened anew when Raskin and Sokoloff (1974) using highly sensitive methods, detected small amounts of alcohol dehydrogenase activity in the soluble fraction of homogenates of whole rat brain and also reported that chronic ingestion of ethanol caused an increase in the alcohol dehydrogenase activity of cerebral tissue. These conflicting results from *in vitro* experiments did not answer the question of whether the intact mammalian brain can metabolize ethanol. It is difficult to obtain unambiguous results concerning this question from experiments in intact animals, because the metabolism of ethanol by the liver is so large and so rapid. In such experiments, one cannot exclude the possibility that acetaldehyde, or acetate produced in the liver from ethanol, was carried to the brain and further metabolized there. For this reason, the isolated, perfused brain was particularly well suited for such experiments, since it provided an intact brain with no interference from liver or other organs.

If [ $^{14}\text{C}$ ]-ethanol were metabolized by the perfused brain, it would be converted to acetaldehyde and then to acetate. Perhaps the most sensitive method to determine whether [ $^{14}\text{C}$ ]-acetate was produced is to measure the incorporation of [ $^{14}\text{C}$ ]-into amino acids in the brain. O'Neal and Koeppe (1966) found that when [ $^{14}\text{C}$ ]-acetate was administered to rats, nearly all the radioactivity in the brain was in the free amino acids. Mukherji and Sloviter (1973) found that when the isolated rat brain was perfused with fluid containing [ $^{14}\text{C}$ ]-acetate, there was considerable incorporation of radioactivity into the free amino acids of the cerebral tissue. Mukherji *et al.* (1975) performed experiments in which the isolated rat brain was perfused with fluid containing [ $^{14}\text{C}$ ]-ethanol, [ $^{14}\text{C}$ ]-acetaldehyde, or [ $^{14}\text{C}$ ]-acetate. After perfusion, the cerebral tissue was rapidly frozen, extracts of the frozen tissue were prepared and the amino acids were separated and isolated by ion-exchange chromatography. The amount and the radioactivity of each amino acid were measured and specific activities were calculated. The results of these experiments are shown in table 3. After perfusion with either [ $^{14}\text{C}$ ]-acetaldehyde or [ $^{14}\text{C}$ ]-acetate, there was considerable incorporation of radioactivity into all the free amino acids of the cerebral tissue. The perfusion with [ $^{14}\text{C}$ ]-acetate was done with 10 times as much radioactivity as was present in the

Table 3. Incorporation of radioactivity into amino acids of isolated perfused rat brain.

	Perfused with 10 $\mu\text{Ci}$ [ $1\text{-}^{14}\text{C}$ ]- acetate (cpm/ $\mu\text{mol}$ )	Perfused with 1 $\mu\text{Ci}$ [ $1,2\text{-}^{14}\text{C}$ ]- acetaldehyde (cpm/ $\mu\text{mol}$ )	Perfused with 5 $\mu\text{Ci}$ [ $1\text{-}^{14}\text{C}$ ]-ethanol (cpm/ $\mu\text{mol}$ )
Glutamine	23,653	1,575	0
Glutamic acid	8,112	1,140	0
Aspartic acid	7,512	2,320	0
GABA	6,595	920	0

perfusion with [ $^{14}\text{C}$ ] acetaldehyde. If the values for radioactivity incorporated from [ $^{14}\text{C}$ ] acetate are divided by 10, the values are of the same order of magnitude as those obtained with [ $^{14}\text{C}$ ] acetaldehyde.

The absence of incorporation of radioactivity from [ $^{14}\text{C}$ ] ethanol into the free amino acids of the brain showed that the perfused rat brain did not metabolize ethanol at a measurable rate. Since there was incorporation of [ $^{14}\text{C}$ ] acetaldehyde and [ $^{14}\text{C}$ ] acetate into the free amino acids, it is apparent that the metabolism of ethanol did not occur because there was no measurable amount of alcohol dehydrogenase activity in the cerebral tissue. The fact that there was incorporation of radioactivity from acetaldehyde indicated that there is aldehyde dehydrogenase activity in cerebral tissue (Erwin and Dietrich, 1966). It does not exclude the possibility that the aldehyde dehydrogenase reaction is a rate-limiting one, since the permeability of the blood-brain barrier is probably greater to acetaldehyde than to acetate. Thus, if aldehyde dehydrogenase activity were rate limiting, the observed approximately equal incorporation might be due to a higher concentration of acetaldehyde than acetate in the cerebral tissue.

### *Insulin*

It is generally believed that insulin does not have a direct effect on the metabolism of the brain; Quastel (1970) has reviewed the evidence for this belief. However, Rafaelsen (1967) has reviewed other experiments with results that have been interpreted as indicating that insulin does have a direct effect on the brain. The isolated perfused brain is well suited to determining whether insulin has a direct effect on the brain. It permits observations on the transport of substances from blood into the brain, which *in vitro* preparations do not. It also eliminates the difficulties of interpretation caused by secondary effects from other organs, which occur in intact animals. Sloviter and Yamada (1971) perfused the isolated rat brain with fluid containing insulin or perfused the brain of a rat previously injected with insulin. The spontaneous electrical activity of the brain, the rate of cerebral consumption of glucose and the rate of efflux of potassium from the brain were not affected by insulin. It was concluded that insulin does not act directly on the brain and that the effects on brain metabolism observed after administering insulin and glucose to the intact animal are probably secondary to the large stimulation of the metabolism of liver and other organs.

### *Dimethyl sulphoxide*

Dimethyl sulphoxide (DMSO) has been shown to alter the permeability of cells and to enhance the transport of physiological substances and drugs across the blood-brain barrier (Hanig *et al.*, 1971). This property of DMSO makes it a potentially valuable agent for the study of psychopharmacologic agents. Ghosh *et al.* (1976) investigated the changes in metabolism of the isolated rat brain during perfusion with fluid containing DMSO. DMSO caused an increase in the rate of glycolysis, a slight decrease in the energy reserves (creatine phosphate and ATP) of the brain and a shift to a more reduced state (increase in lactate/pyruvate ratio) in the brain tissue. It was suggested that the observed effects were probably due to an inhibitory action

Variable results have been obtained in studies of the effects of the acute administration of morphine on the metabolism and energy reserves of brain tissue (Veech *et al.*, 1973). Mukherji *et al.* (1980) studied the effects of morphine and also of methadone on the cerebral cortex of the isolated, perfused rat brain. Perfusion of the isolated rat brain with fluid containing morphine (50  $\mu\text{g/ml}$ ) caused increases in both glucose consumption and lactate production; perfusion with methadone (50  $\mu\text{g/ml}$ ) caused an increase in glucose consumption but no increase in lactate production. Perfusion with morphine caused a marked decrease in the energy reserves of the brain tissue but perfusion with methadone caused only a slight decrease. The results suggested that morphine may inhibit mitochondrial oxidative activity.

## Oxidation-reduction state

Since the perfluoro compound, used in place of red blood cells in the perfusion fluid, has no optical absorption or fluorescence, its use avoids the interference in optical measurements caused by haemoglobin. Thus, in the perfused brain preparation, which contained no haemoglobin, it was possible to determine the redox state of pyridine nucleotides (NADH) in cerebral cortex by fluorescence measurements using quartz fiber optics. It was found that increase in fluorescence from NADH caused by anoxia was about the same in the perfused brain as in the brain of the intact rat (Mayevsky *et al.*, 1981).

## References

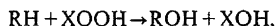
- Andjus, R. K., Suhara, K. and Sloviter, H. A. (1967) *J. Appl. Physiol.*, **22**, 1033.  
 Beer, C. T. and Quastel, J. H. (1958) *Can. J. Biochem. Physiol.*, **36**, 531.  
 Bouckaert, A. J. and Jourdan, F. (1956) *Arch. Int. Pharmacodyn. Ther.*, **53**, 523.  
 Chute, A. L. and Smyth, D. H. (1939) *Q. J. Exp. Physiol.*, **29**, 379.  
 Cremer, J. E. (1970) *Biochem. J.*, **119**, 95.  
 Elliott, K. A. C., Scott, D. B. M. and Libet, B. (1942) *J. Biol. Chem.*, **146**, 251.  
 Erwin, V. G. and Dietrich, R. A. (1966) *J. Biol. Chem.*, **241**, 3533.  
 Geiger, A. (1958) *Physiol. Rev.*, **38**, 1.  
 Geiger, A. and Magnes, J. (1947) *Am. J. Physiol.*, **149**, 517.  
 Ghosh, A. K., Mukherji, B. and Sloviter, H. A. (1972) *J. Neurochem.*, **19**, 1279.  
 Ghosh, A. K., Ito, T., Ghosh, S. and Sloviter, H. A. (1976) *Biochem. Pharmacol.*, **25**, 1115.  
 Hanig, J. P., Morrison, J. M. and Krop, S. (1971) *J. Pharm. Pharmacol.*, **23**, 386.  
 Heymans, J. F. and Kochmann, M. (1904) *Arch. Int. Pharmacodyn. Ther.*, **13**, 379.  
 Maddock, S., Hawkins, J. E. and Holmes, E. (1939) *Am. J. Physiol.*, **125**, 551.  
 Mayevsky, A., Mizawa, I. and Sloviter, H. A. (1981) *Neurol. Res.*, **3**, 307.  
 Moss, G. (1964) *J. Surg. Res.*, **4**, 170.  
 Mukherji, B. and Sloviter, H. A. (1973) *J. Neurochem.*, **20**, 633.  
 Mukherji, B., Turinsky, J. and Sloviter, H. A. (1971) *J. Neurochem.*, **18**, 1783.  
 Mukherji, B., Kashiki, Y., Ohyanagi, H. and Sloviter, H. A. (1975) *J. Neurochem.*, **24**, 841.  
 Mukherji, B., Suemaru, K., Sakai, N., Ghosh, A. K. and Sloviter, H. A. (1980) *Biochem. Pharmacol.*, **29**, 1608.  
 O'Neal, R. M. and Koeppe, R. E. (1966) *J. Neurochem.*, **13**, 835.  
 Quastel, J. H. (1970) *Neurosci. Res.*, **3**, 1.  
 Rafaelsen, O. J. (1967) *Acta. Med. Scand. (Suppl.)*, **476**, 75.

- Sacks, W. (1961) *Handbook Neurochem.*, **1**, 301.
- Schmidt, C. F. (1928) *Am. J. Physiol.*, **84**, 202.
- Sloviter, H. A. and Kamimoto, T. (1967) *Nature (London)* **216**, 458.
- Sloviter, H. A. and Yamada, H. (1971) *J. Neurochem.*, **18**, 1269.
- Smith, A. L. and Wollman, H. (1972) *Anesthesiology*, **36**, 378.
- Tallan, H. H., Moore, S. and Stein, W. J. (1954) *J. Biol. Chem.*, **211**, 927.
- Thompson, A. M., Robertson, R. C. and Bauer, T. A. (1968) *J. Appl. Physiol.*, **24**, 407.
- Veech, R. L., Harris, R. L., Veloso, D. and Veech, E. H. (1973) *J. Neurochem.*, **20**, 183.
- White, R. J., Albin, M. S. and Verdura, J. (1964) *Nature (London)*, **202**, 1082.



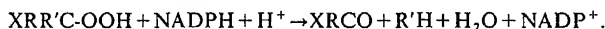
**MINOR J. COON and ALFIN D. N. VAZ**

Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109, USA

**Abstract.** Highly purified liver microsomal cytochrome P-450 acts as a peroxygenase in catalyzing the reaction,

where RH represents any of a large variety of foreign or physiological substrates and ROH the corresponding product, and XOOH represents any of a series of peroxy compounds such as hydroperoxides or peracids serving as the oxygen donor and XOH the resulting alcohol or acid. Several experimental approaches in this and other laboratories have yielded results compatible with a homolytic mechanism of oxygen-oxygen bond cleavage but not with the heterolytic formation of a common iron-oxo intermediate from the various peroxides.

Recently, we have found a new reaction, catalyzed by the reconstituted system containing the phenobarbital-inducible form of P-450, which catalyzes the reductive cleavage of hydroperoxides:



Thus, cumyl hydroperoxide yields acetophenone and methane, and 13-hydroperoxyoctadeca-9,11-dienoic acid yields pentane and an as yet unidentified additional product. Since hydroperoxide reduction does not produce the corresponding alcohol, it is concluded that homolytic cleavage of the oxygen-oxygen bond occurs with rearrangement of the resulting alkoxy radical. Studies are in progress to determine how broad a role the new hydroperoxide cleavage reaction plays in the biological peroxidation of lipids.

**Keywords.** Cytochrome P-450; peroxides; oxygen radicals; lipid peroxidation; hydroxylation reactions; hydroperoxides.

## Introduction

As is now widely known, the term cytochrome P-450 is used to refer to a group of heme proteins that are apparently unique in having a sulphur atom ligated to the iron and that form carbon monoxide complexes that have a major absorption band at about 450 nm (White and Coon, 1980). Cytochrome P-450 catalyzes not only aliphatic and aromatic hydroxylation, but also N-oxidation, sulfoxidation, epoxidation, N-, S-, and O-dealkylation, peroxidation, deamination, desulphuration, and dehalogenation, but also reactions such as reduction of azo groups, nitro groups, N-oxides, and epoxides that involve only electron transfer and partially justify the term 'cytochrome' for this enzyme. The substrates include physiologically occurring lipids such as fatty acids, prostaglandins, and steroids, as well as a host of foreign compounds including drugs, petroleum products, anesthetics, insecticides, and carcinogens. In recent years, cytochrome P-450 has been purified to apparent homogeneity from numerous sources, and from the primary structures established by protein and DNA sequencing it is clear that the various forms of P-450 are distinct

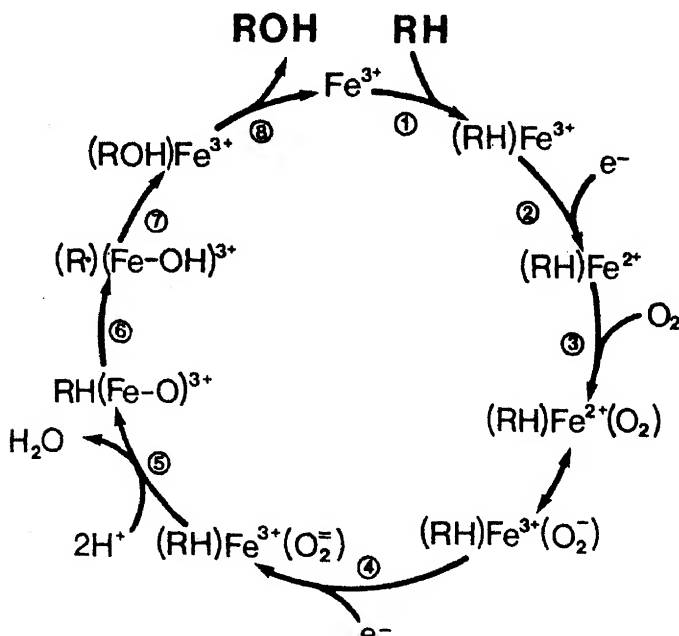


gene products (Black and Coon, 1986). Progress has also been made in understanding the regulation of P-450 gene expression; for example, Ravishankar and Padmanaban (1985) have proposed that heme is a general regulator of expression at the level of transcription, whereas a particular drug or its metabolite would impart the specificity needed for the induction of a particular form of the cytochrome. The various forms or isozymes of P-450 participate in metabolically important transformations of lipids and also catalyze the alteration of xenobiotics in ways that usually lead to detoxification but in some instances yield products with greater cytotoxic, mutagenic, or carcinogenic properties.

The purpose of this communication is to provide a brief review of our mechanistic studies on the role of peroxides in P-450-catalyzed reactions. Emphasis is placed on evidence for radical species derived from hydroperoxides in P-450-dependent reactions.

*Catalytic cycle for oxygen-dependent hydroxylation reactions catalyzed by cytochrome P-450*

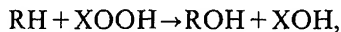
As background for our recent investigations on peroxides, the scheme we have proposed for  $O_2$ -dependent reactions, based on findings in this and numerous other laboratories, is given in figure 1. The overall scheme is in accord with the known stoichiometry of such reactions (Gorsky *et al.*, 1984) and also takes into account the known regioselectivity and partial loss of configuration during oxidation of prochiral



The steps are as follows. (i) Substrate binding, in which the principal contributor to the binding energy is probably the hydrophobic effect. (ii) First electron transfer, the primary donor being the FMN- and FAD-containing reductase in the case of the microsomal system and an iron-sulphur redoxin in the case of the bacterial and mitochondrial hydroxylation systems. (iii) Dioxygen binding to give a ferrous dioxygen complex with sufficient stability to be detectable by Mössbauer and stopped flow spectrophotometry; a resonance form, the ferric-superoxide species, serves as the source of the superoxide radical coming from this 'leaky' enzyme system. (iv) Second electron transfer, in which cytochrome  $b_5$  sometimes substitutes effectively for the P-450 reductase (Pompon and Coon, 1984) and the product is at the redox level of hydrogen peroxide. (v) Splitting of the oxygen-oxygen bond to yield water and an iron-oxene species. (vi) Hydrogen abstraction from the substrate (Groves *et al.*, 1978). (vii) Recombination of the substrate carbon radical and the proposed hydroxyl radical species to give the product, ROH. (viii) dissociation of the product with recovery of the cytochrome in the resting, ferric state.

#### *Catalytic cycle for peroxide-dependent hydroxylation reactions catalyzed by cytochrome P-450*

In addition to catalyzing various  $O_2$ -dependent reactions, P-450 also brings about the oxygenation of organic substrates at the expense of peroxy compounds. This reaction, first shown with drugs and cumyl hydroperoxide in microsomal suspensions (Kadlubar *et al.*, 1973; Rahimtula and O'Brien, 1974), occurs with a number of oxidants other than alkyl hydroperoxides (such as peroxyacids, sodium periodate, iodosobenzene, iodosobenzene diacetate, and N-oxides) and involves cytochrome P-450, as reviewed elsewhere (Black and Coon, 1986b). This laboratory showed that electrophoretically homogeneous P-450 isozyme 2 catalyzes such reactions in the absence of NADPH, NADPH-cytochrome P-450 reductase, and molecular oxygen and that the peroxygenase reaction has the following stoichiometry:



where RH and ROH again represent the substrate and product, respectively, and XOOH is the peroxy compound serving as oxygen donor. A difference has been noticed between peroxide-based oxidants and their iodine-based counterparts. Specifically, with peroxides the oxygen atom incorporated into the product alcohol is derived exclusively from the peroxide (Nordblom *et al.*, 1976), whereas the oxygen atom in alcohols formed in the presence of agents such as iodosobenzene appears to be derived from water (Heimbrook and Sligar, 1981; White and McCarthy, 1984) because of a solvent exchange reaction (White, 1986). Thus, the various available oxidants may not all be equally good mechanistic models.

The peroxide shunt is shown in figure 2. Several experimental approaches were used in this laboratory in an attempt to determine whether peroxide oxygen-oxygen bond cleavage occurs by a homolytic or heterolytic mechanism, as summarized elsewhere (Black and Coon, 1987; Coon and Blake, 1982). The reaction of rabbit P-

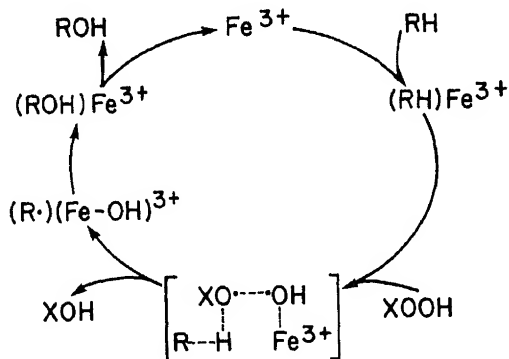
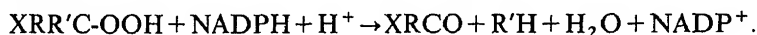


Figure 2. Peroxide shunt, in which a peroxy compound, XOOH, serves as the oxygen donor to the substrate.

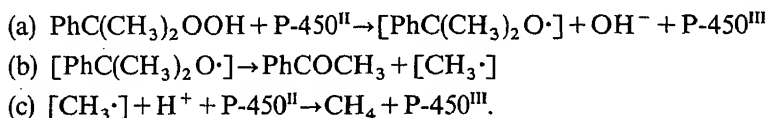
450 isozyme 2 with peroxides differs in several important respects from that of peroxidases. In the case of P-450 only, the intermediates are formed reversibly, and their spectra vary with structural differences in the peroxy compounds (Blake and Coon, 1980). The absolute spectra as well as the difference spectra with the various peroxides differ not only in the magnitude but also in the positions of the maxima and minima. Secondly, several P-450's were shown to bring about the decarboxylation of peroxyphenylacetic acid in a reaction presumably involving the phenylacetoxo radical as an intermediate (White *et al.*, 1980). Finally, studies with a series of *m*- and *p*-substituted derivatives of cumyl hydroperoxide as the oxygen donor and of toluene as the substrate showed that the hydroxylation rate constant is sensitive to alterations in both reactants (Blake and Coon, 1981). The reaction of toluene with cumyl hydroperoxide to form benzyl alcohol and cumyl alcohol was followed by stopped flow spectrophotometry; the two spectral complexes formed were examined, along with the rate of benzyl alcohol formation in the presence of 3-acetylpyridine adenine dinucleotide and alcohol dehydrogenase. In such studies a number of *m*- and *p*-substituted toluenes and cumyl hydroperoxides were employed. Additional work by McCarthy and White (1983) established that peroxy acid decarboxylation is unique to P-450 enzymes. Thus, whereas all P-450's examined have been found catalytically competent in decarboxylation, other heme proteins, including peroxidases, promote only heterolytic cleavage. The 3 dimensional crystal structure of bacterial P-450<sub>cam</sub> (Poulos *et al.*, 1985) reveals that this enzyme has none of the general acid-base and charge stabilization machinery normally considered necessary for heterolytic oxygen-oxygen bond cleavage. In summary, these results are compatible with a homolytic mechanism of oxygen-oxygen bond cleavage but not with the heterolytic formation of a common iron-oxo intermediate from the various peroxides, as generally seen with peroxidase-catalyzed reactions.

*NADPH oxidation by hydroperoxides: Catalysis by cytochrome P-450 and evidence for homolytic cleavage of the peroxide bond*

we have found that simple 2-electron transfer to cumyl hydroperoxide to yield the corresponding alcohol does not occur; acetophenone was produced instead (Vaz and Coon, 1985). The other product was then identified as methane by GC-mass spectrometry (Vaz and Coon, 1986), and the stoichiometry was shown to correspond to the following general reaction for the reductive cleavage of peroxides:



The data in table 1 show that the hydroperoxide-dependent NADPH oxidation requires both P-450 and the reductase and that the native enzymes are necessary for activity. In other experiments the addition of benzphetamine had no effect, whereas carbon monoxide inhibited the reaction about 65%. Our current view of the mechanism of the reaction is that alkyl hydroperoxides are reduced stepwise as shown here for cumyl hydroperoxide (Vaz and Coon, 1985):



Homolytic cleavage of the oxygen-oxygen bond with transfer of one electron from P-450 is pictured as yielding the cumyloxy radical and hydroxide ion; the radical rearranges to give acetophenone and a transient methyl radical, and the latter with uptake of the second electron produces methane.

**Table 1.** Requirements for cumyl hydroperoxide-dependent NADPH oxidation<sup>a</sup>.

System	NADPH oxidation (nmol/min/nmol P-450)
Complete	30.0
P-450 omitted	0.1
Reductase omitted	3.6
P-450 and reductase omitted	0.1
P-450 heat-inactivated	0.8
Reductase heat-inactivated	3.6

<sup>a</sup>Taken from Vaz and Coon (1985). The complete system contained P-450 form 2 (0.05  $\mu\text{M}$ ), NADPH-cytochrome P-450 reductase (0.05  $\mu\text{M}$ ), dilauroylglyceryl-3-phosphorylcholine (30  $\mu\text{g/ml}$ ), and cumyl hydroperoxide (0.4 mM) in 0.05 M potassium phosphate buffer, pH 7.4, containing EDTA (10 mM). For inactivation, the enzyme was boiled for 5 min. The incubations were at 25°C.

In addition to cumyl hydroperoxide,  $\alpha$ -methylbenzyl hydroperoxide, benzyl hydroperoxide, and *t*-butyl hydroperoxide also yield products in accord with the reductive cleavage already shown. Furthermore, we have added 13-hydroperoxy-octadeca-9,11-dienoic acid to the reconstituted system containing phenobarbital-inducible P-450 form 2, the reductase, and NADPH, and have identified pentane as a product as well as a compound not yet fully characterized but having the properties

attempt will be made to review here the biochemical literature on microsomal lipid peroxidation, other than to indicate that NADPH-cytochrome P-450 reductase is usually regarded as the chief source of oxygen species that initiate the conversion of polyunsaturated fatty acids to hydroperoxides (Aust *et al.*, 1972; Lai *et al.*, 1979). The role of cytochrome P-450 in lipid peroxidation has been the subject of investigation but remains uncertain (Hrycay and O'Brien, 1971; Svingen *et al.*, 1979; Ekström and Ingelman-Sundberg, 1984). Although much remains to be learned, our present findings with phenobarbital-inducible rabbit liver microsomal P-450 form 2 and with model hydroperoxides as well as a hydroperoxide derived from linoleic acid establish a new reaction catalyzed by this cytochrome. The reductive cleavage of lipid hydroperoxides by P-450 would account for many of the final products that are believed to arise biologically, including aldehydes and alkyl hydrocarbons (Wendel and Dumelin, 1981).

## Acknowledgement

This research was supported by Grant DK-10339 from the National Institutes of Health, USA.

## References

- Aust, S. D., Roerig, D. L. and Pederson, T. C. (1972) *Biochem. Biophys. Res. Commun.*, **47**, 1133.
- Black, S. D. and Coon, M. J. (1986) in *Cytochrome P-450* (ed. P. R. Ortiz de Montellano) (New York: Plenum Press) p. 161.
- Black, S. D. and Coon, M. J. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.*, (in press).
- Blake, R. C., II and Coon, M. J. (1980) *J. Biol. Chem.*, **255**, 4100.
- Blake, R. C., II and Coon, M. J. (1981) *J. Biol. Chem.*, **256**, 12127.
- Coon, M. J. and Blake, R. C., II (1982) in *Oxygenases and Oxygen Metabolism* (eds M. Nozaki, S. Yamamoto, Y. Ishimura, M. J. Coon, L. Ernster and R. W. Estabrook) (New York: Academic Press) p. 485.
- Coon, M. J. and Vaz, A. D. N. (1986) *Chem. Scr.*, (in press).
- Ekström, G. and Ingelman-Sundberg, M. (1984) *Biochem. Pharmacol.*, **33**, 2521.
- Gorsky, L. D., Koop, D. R. and Coon, M. J. (1984) *J. Biol. Chem.*, **259**, 6812.
- Groves, J. T., McClusky, G. A., White, R. E. and Coon, M. J. (1978) *Biochem. Biophys. Res. Commun.*, **81**, 154.
- Heimbrook, D. C. and Sligar, S. G. (1981) *Biochem. Biophys. Res. Commun.*, **99**, 530.
- Hrycay, E. G. and O'Brien, P. J. (1971) *Arch. Biochem. Biophys.*, **147**, 14.
- Kadlubar, F. F., Marton, K. C. and Ziegler, D. M. (1973) *Biochem. Biophys. Res. Commun.*, **54**, 1255.
- Lai, C.-S., Grover, T. A. and Piette, L. H. (1979) *Arch. Biochem. Biophys.*, **193**, 373.
- McCarthy, M. B. and White, R. E. (1983) *J. Biol. Chem.*, **258**, 9153.
- Nordblom, G. D., White, R. E. and Coon, M. J. (1976) *Arch. Biochem. Biophys.*, **175**, 524.
- Pompon, D. and Coon, M. J. (1984) *J. Biol. Chem.*, **259**, 15377.
- Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C. and Kraut, J. (1985) *J. Biol. Chem.*, **260**, 16122.
- Rahimtula, A. D. and O'Brien, P. J. (1974) *Biochem. Biophys. Res. Commun.*, **60**, 440.
- Ravishanker, H. and Padmanaban, G. (1985) *J. Biol. Chem.*, **260**, 1588.
- Svingen, B. A., Buege, J. A., O'Neal, F. O. and Aust, S. D. (1979) *J. Biol. Chem.*, **254**, 5892.
- Vaz, A. D. N. and Coon, M. J. (1985) in *Cytochrome P-450, Biochemistry, Biophysics and Induction* (eds L. Vereczkey and K. Magyar) (Elsevier, Amsterdam, and Akademiai, Kiado, Budapest) p. 545.
- Vaz, A. D. N. and Coon, M. J. (1986) *Fed. Proc.*, **45**, 1871.
- Wendel, A. and Dumelin, E. E. (1981) *Methods Enzymol.*, **77**, 10.
- White, R. E. (1986) *Fed. Proc.*, **45**, 1747.

## Oligosaccharide structure determination of glycoconjugates using lectins

DEBKUMAR BASU\*, JYOTI V. NAIR and P. S. APPUKUTTAN

Neurochemistry Department, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum 695 011, India

**Abstract.** Lectins, the divalent or polyvalent (glyco) proteins of non-immune origin of the cells agglutinate cells or other materials, that display more than one saccharide of sufficient complementarity. Lectins considered 'identical' in terms of mono- and disaccharide specificity can be differentiated by their ability to recognise the fine differences in more complex structures. The present review discusses the interaction of lectins with various oligosaccharides and their resultant separations due to structural variations.

**Keywords.** Oligosaccharide structures; lectins; glycoconjugates.

Lectins, the divalent or multivalent (glyco) proteins of the cells are of non-immune origin. Proteins of this class share a common ability to agglutinate cells or other materials that display more than one saccharide of sufficient complementarity. Such agglutinins are found predominantly in seeds of plants, in particular those of legumes, but are also present in other parts of plants and other living organisms inclusive of human. There are excellent reviews on the isolation, cell agglutination and other functions of lectins (Sharon and Lis, 1972, 1973; Barondes, 1981, 1984). Lectins considered 'identical' in terms of monosaccharide specificity possess the ability to recognise fine differences in more complex structures. In this review we are discussing the interaction of lectins with various glycoproteins, glycopeptides and their separation due to oligosaccharide structures.

The presence of Concanavalin A (ConA) in the seeds of *Canavalia ensiformis* was demonstrated by Sumner and Howell (1936). The homogeneous purification and the utilisation of affinity chromatography as technique was achieved by Agarwal and Goldstein (1967). Since then several lectins had been purified and characterised. The statement 'nothing is known about their role in nature' by Lis and Sharon (1973) has been upgraded in recent times (Barondes, 1981, 1984). The aspect of the function of lectin as analytical tool in affinity chromatography has also emerged mostly from the work of Stuart Kornfeld and his co-workers at Washington University, St. Louis, USA. Oligosaccharide structures mentioned in the text are given in tables 1-3.

ConA is the most widely studied lectin. The structural requirements of mono- and disaccharides had been described (Goldstein and Hayes 1978; Brewer and Brown 1979; Loontjens *et al.*, 1983). However, its binding affinity to oligosaccharides seems to depend significantly on the type of linkages and immediate neighbouring saccharides.

---

\*To whom all correspondence should be addressed.

Abbreviation	Structure
M-1	$\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
M-2	$\alpha$ -Man-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
M-3	$\alpha$ -Man-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
N-1	$\alpha$ -NeuAc-(2 $\rightarrow$ 3)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
N-2	$\alpha$ -NeuAc-(2 $\rightarrow$ 6)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
N-3	$\alpha$ -NeuAc-(2 $\rightarrow$ 6)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
N-4	$\alpha$ -NeuAc-(2 $\rightarrow$ 6)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\alpha$ -Man-(1 $\rightarrow$ 6)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
N-5	$\alpha$ -NeuAc-(2 $\rightarrow$ 6)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 6)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
N-6	$\alpha$ -NeuAc-(2 $\rightarrow$ 6)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 6)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
S-5a	$\alpha$ -NeuAc-(2 $\rightarrow$ 6)- $\beta$ -Gal-(1 $\rightarrow$ 4)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 6)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
S-4b	$\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 6)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
	$\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -GlcNAc-(1 $\rightarrow$ 4)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc
	$\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 6)- $\beta$ -GlcNAc-(1 $\rightarrow$ 2)- $\alpha$ -Man-(1 $\rightarrow$ 3)- $\beta$ -Man-(1 $\rightarrow$ 4)-GlcNAc

The lectins with specificity for monosaccharide,  $\alpha$ -mannose and  $\alpha$ -glucose are ConA, and the agglutinins from lentil (*Lens culinaris*), *Vicia faba*, and pea (*Pisum sativum*). ConA presents a great affinity for trimannosidic core substituted by two N-acetyl glucosamine (glcNAc) residues (S-5a). The affinity is not decreased by the addition of a  $\beta$ (1 $\rightarrow$ 4) linked glcNAc linked to  $\beta$ -man residue (S-4b). But the affinity is reduced when these glcNAc-residues are substituted by galactose (gal)-(N-4, N-5) or by  $\beta$ -gal- $\alpha$ (2 $\rightarrow$ 6) N-acetylneuraminic acid (NeuAc)-residues (N-6). But an  $\alpha$ (1 $\rightarrow$ 6) mannose (man) residue substituted  $\beta$ -man core (N-3) was more inhibitory than the core itself (N-2). The oligosaccharides (M-1) without the trimannosidic core even with a terminal  $\alpha$ (1 $\rightarrow$ 3)-man was a poor inhibitor. The addition of even one or two  $\alpha$ (1 $\rightarrow$ 2)-man did increase inhibitory capacity. But an oligosaccharide structure containing the trimannosidic core with  $\alpha$ (1 $\rightarrow$ 3) and  $\alpha$ (1 $\rightarrow$ 6)-man was a good inhibitor. The substitution of the core  $\alpha$ (1 $\rightarrow$ 3)-man at C-4 position (OVO-TF) made it a poor inhibitor. Lentil, *Vicia faba* and Pea lectins showed best inhibition when Asparagine (Asn)-linked first  $\beta$ -glcNAc was substituted with  $\alpha$ (1 $\rightarrow$ 6)-fucose (fuc) residue (hLTF 1 and 2). The removal of  $\alpha$ -fuc residue made it a poor inhibitor (Debray *et al.*, 1981). Similar role of  $\alpha$ -fuc residue attached to Asn-linked  $\beta$ -glcNAc of the core was observed by Kornfeld *et al.* (1981). They also showed that sialylated rat immunoglobulin E and mouse immunoglobulin M (1B), glycopeptides bind to immobilised pea and lentil lectins and eluted only with 500 mM  $\alpha$ -methyl mannoside in the eluting buffer. Lentil lectin has a hydrophobic region in or near the saccharide binding site as shown by Allen *et al.* (1976), since 3-O-methyl- and 3-O-benzyl derivative D-glucose (glc) was found to be superior inhibitors to D-glc alone. This idea was supported by Debray *et al.* (1981) on account of the presence of a fucose at Asn

Table 2. Oligosaccharide structures.

Abbreviation	Structure
GP-hSTF	$\alpha\text{-NeuAc-(2}\rightarrow\text{6)-}\beta\text{-Gal-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{3)-}\beta\text{-Man-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{3)-Asn}$
GP-hLTF-1	$\alpha\text{-NeuAc-(2}\rightarrow\text{6)-}\beta\text{-Gal-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{6)-}$
	$\alpha\text{-NeuAc-(2}\rightarrow\text{6)-}\beta\text{-Gal-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{3)-}$
	$\alpha\text{-NeuAc-(2}\rightarrow\text{6)-}\beta\text{-Gal-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{6)-}\beta\text{-Man-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{3)-Asn}$
GP-hLTF-2	$\alpha\text{-NeuAc-(2}\rightarrow\text{6)-}\beta\text{-Gal-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{3)-}$
	$\beta\text{-Gal-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{6)-}\beta\text{-Man-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{3)-Asn}$
GP-ovoTF	$\beta\text{-GlcNAc-(1}\rightarrow\text{4)-}$
	$\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{3)-}$
	$\beta\text{-GlcNAc-(1}\rightarrow\text{2)-}\alpha\text{-Man-(1}\rightarrow\text{6)-}\beta\text{-Man-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{4)-}\beta\text{-GlcNAc-(1}\rightarrow\text{3)-Asn}$



Table 3. Oligosaccharide structures.

S.A $\alpha 2,3(6) \downarrow$ Gal $\beta 1,4 \downarrow$ GlcNAc $\beta 1,2 \downarrow$ Man $\alpha 1,3 \swarrow$	S.A $\alpha 2,3(6) \downarrow$ Gal $\beta 1,4 \downarrow$ GlcNAc $\beta 1,2 \downarrow$ Man $\alpha 1,6 \swarrow$	S.A $\alpha 2,6 \downarrow$ Gal $\beta 1,4 \downarrow$ GlcNAc $\beta 1,2 \downarrow$ Man $\alpha 1,3(6) \swarrow$	Gal $\beta 1,4 \downarrow$ GlcNAc $\beta 1,2 \downarrow$ Man $\alpha 1,6(3) \swarrow$
Man $\beta 1,4 \downarrow$ GlcNAc $\beta 1,4 \downarrow$ Fuc $\xrightarrow{\alpha 1,6}$ GlcNAc Asn Rat IgE		Man $\beta 1,4 \downarrow$ GlcNAc $\beta 1,4 \downarrow$ Fuc $\xrightarrow{\alpha 1,6}$ GlcNAc Asn Mouse IgM (1B)	

linked glcNAc. It has also been observed that removal of NeuAc- and gal-from the biantennary oligosaccharide chains made it poor inhibitor, but removal of NeuAc alone had no effect (h-STE). Lentil and *Vicia faba* agglutinins were not inhibited by oligosaccharides with terminal man-residues in a terminal non-reducing position (M1-M3). The absence of fuc at Asn-N-acetyl glucosamine (glcNAc) (N-6) residue made it 130-times less inhibitory (hLTF1 and 2) to lentil lectin. It also showed greater affinity for oligosaccharides with substitution by NeuAc residue at C-6 position of gal rather than at C-3 (N1, N2 and N4). Lentil, pea and *Vicia faba* agglutinins had an hydrophobic area in or near carbohydrate binding site and this site could cause non-specific interaction between lectins and aromatic residues of glycoproteins or glycopeptides (Debray *et al.*, 1981). Kornfeld *et al.* (1981) had stated that the above mentioned lectins had closely related primary structure and subunit assembly, hence it was not unexpected that they showed similarity in saccharide-binding specificity.

*Ricinus communis* agglutinin (RCAI) and *R. communis* toxin (RCAII) have different molecular weights, 120 kDa and 60 kDa, respectively. RCAI showed greater affinity for saccharide sequences possessing gal-residue(s) substituted by NeuAc residue(s) at C-6 rather than at C-3 position (N1, N2, N4). The above observation was made by Baenziger and Fiete (1979) and had been confirmed by Debray *et al.* (1981). Adair and Kornfeld (1974) had observed that human immunoglobulin G glycan structure was identical to that of a glycopeptide of human lactotransferrin and was 3-fold more inhibitory than the glycopeptide of human transferrin which lacked  $\alpha(1 \rightarrow 6)$  fuc near N-glycosidic linkage. Baenziger and Fiete (1979) observed that RCAII had better affinity towards O-glycosidic linkages with  $\beta$ -gal (1 $\rightarrow$ 3)-galNAc sequence. RCAII showed higher affinity than RCAI when similar sequence was present in N-glycosidic linkages.

Both RCA lectin and Winged bean agglutinin (WBA) are inhibited by galNAc and simple  $\beta$ -galactosides, like lactose. However, the strong affinity of  $\beta$ -gal terminating

(1→4)- $\beta$ -glcNAc (1→3)-Asn and  $\beta$ -glcNAc (1→4)- $\beta$ -glcNAc (1→3)-Asn with equivalent inhibitory power to chitobiose. WGA was found to interact with NeuAc of glycoconjugates (Bhavanandan and Katlic, 1973; Peters *et al.*, 1979). Monsigny *et al.* (1980) had termed this interaction as a 'charge effect'. It had been observed that glycopeptides containing two external glcNAc residues and their asialoagalacto form with two exposed glcNAc residues were not inhibitory to WGA agglutination. However, Yamamoto *et al.* (1981) observed with ovalbumin glycopeptides that the N,N'-diacetyl chitobiose moiety and  $\beta$ -glcNAc residue linked to  $\beta$ -man contributed to the interaction of the glycopeptides with WGA-Sepharose.

*Phaseolus vulgaris* (PHA) contain two lectins one agglutinated erythrocytes (E-PHA) and the other agglutinated leukocytes (L-PHA). High affinity binding to E-PHA occurs only with biantenary glycopeptides with 2 outer gal-residues and a GlcNAc-residue linked  $\beta$ (1→4) to  $\beta$ -man of the trimannosidic core. The same species did not bind to L-PHA. Tri- and tetraantenary glycopeptides containing outer gal-residues and an  $\alpha$ -linked man substituted at positions C-2 and C-6 are specifically required for L-PHA (Cummings and Kornfeld, 1982).

Cummings and Kornfeld (1982) had isolated and demonstrated the structures of high man, hybrid and complex types of glycopeptides from mouse lymphoma cells (BW 5147) by serial fractionation through immobilised ConA, WGA, Pea, E-PHA and L-PHA columns. These glycopeptides were all N-asparaginyl linked. ConA bound eluted with 100 mM  $\alpha$ -methyl mannoside at 60°C contained high man and hybrid type oligosaccharides. These can be further separated by passing through WGA column into unbound high man and bound hybrid type. ConA unbound and 10 mM  $\alpha$ -methyl mannoside eluate at 25°C contained various types of complex oligosaccharides. They were resolved to near homogeneity by affinity chromatography on pea, E-PHA and L-PHA columns.

Soluble oligosaccharide sequence preferences among naturally occurring glycoproteins for lectins may not be apparent in their affinity for mono-or disaccharide specificities. Thus both *Artocarpus integrifolia* (Jacalin) and *Griffonia simplicifolia* lectins prefer  $\alpha$ -linked galactose containing mono-or disaccharides for agglutination inhibition (Sureshkumar *et al.*, 1982; Murphy and Goldstein, 1977, 1979). However the marked affinity of Jacalin for immunoglobulin oligosaccharide sequence is absent in *griffonia* lectin (Roque-Barreira and Campos-Neto, 1985). Notably the suggested structure of immunoglobulin did not contain  $\alpha$ -gal residues (Baenziger and Kornfeld, 1974).

## Acknowledgements

The work carried out in this laboratory has been supported by grants from Council of Scientific and Industrial Research and Department of Science and Technology, New Delhi.

## References

- Adair, W. L. and Kornfeld, S. (1974) *J. Biol. Chem.*, **249**, 4696.
- Agarwal, B. B. L. and Goldstein, I. J. (1967) *Biochim. Biophys. Acta*, **147**, 262.
- Allen, A. K., Neuberger, A. and Sharon, N. (1973) *Biochem. J.*, **131**, 155.

- Allen, A. K., Desai, N. N. and Neuberger, A (1976) *Biochem. J.*, **155**, 127.
- Baenziger, J. U. and Kornfeld, S. (1974) *J. Biol. Chem.*, **249**, 7260, 7270.
- Baenziger, J. U. and Fiete, D. (1979) *J. Biol. Chem.*, **254**, 2400.
- Barondes, S. H. (1981) *Annu. Rev. Biochem.*, **50**, 207.
- Barondes, S. H. (1984) *Science*, **227**, 1259.
- Bhavanandan, V. P. and Katlic, A. W. (1979) *J. Biol. Chem.*, **254**, 4000.
- Brewer, C. F. and Brown, R. D. (1979) in *Carbohydrate—Protein Interactions* (ed. I. J. Golds) (Washington D. C.: American Chemical Society) p. 27.
- Cummings, R. D. and Kornfeld, S. (1982) *J. Biol. Chem.*, **257**, 11230, 11235.
- Debray, H., Decont, D., Strecker, G., Spik, G. and Montreuil, J. (1981) *Eur. J. Biochem.*, **117**, 41.
- Goldstein, I. J., Hummerström, S. and Lundblad, G. (1975) *Biochim. Biophys. Acta*, **405**, 53.
- Goldstein, I. J. and Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.*, **35**, 127.
- Kornfeld, K., Reitman, M. L. and Kornfeld, R. (1981) *J. Biol. Chem.*, **256**, 6633.
- Lis, H. and Sharon, N. (1973) *Annu. Rev. Biochem.*, **42**, 541.
- Loontjens, F. G., Clegg, R. M. and Landschoot, A. V. (1983) *J. Biosci.*, **5**, 105.
- Monsigny, M., Delmotte, M. and Helene, C. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1324.
- Monsigny, M., Roche, A. C., Sene, C., Maget-Dana, R. and Delmotte, M. (1980) *Eur. J. Biochem.*, **104**, 1.
- Murphy, L. A. and Goldstein, I. J. (1977) *J. Biol. Chem.*, **252**, 4739.
- Murphy, L. A. and Goldstein, I. J. (1979) *Biochemistry*, **18**, 4999.
- Peters, B. P., Ebisu, S., Goldstein, I. J. and Flashner, M. (1979) *Biochemistry*, **18**, 5505.
- Roque-Barreira, M. C. and Campos-Neto, A. (1985) *J. Immunol.*, **134**, 1740.
- Sharon, N. and Lis, H. (1972) *Science*, **177**, 949.
- Sumner, J. B. and Howell, S. E. (1936) *J. Bacteriol.*, **32**, 227.
- Sureshkumar, G., Appukuttan, P. S. and Basu, D. (1982) *J. Biosci.*, **4**, 257.
- Yamamoto, K., Tsuji, T., Matsumoto, I. and Osawa, T. (1981) *Biochemistry*, **20**, 5894.

# Molecular biology of Ri-plasmid—A review

VILAS P. SINKAR, FRANK F. WHITE\*, and MILTON P. GORDON

Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA

\*Department of Plant Pathology, Kansas State University, Manhattan, Kansas 66506, USA

**Abstract.** *Agrobacterium rhizogenes* transfers a segment of its plasmid to the plant genome. The transferred DNA contains genes which are involved in the synthesis of plant hormones. These genes express in the plant cell and give rise to rooty-tumors at the infection site. Transgenic plants can be readily regenerated from the rooty-tumors and the transferred DNA is transmitted to progeny plants. High regeneration potential and sustained maintenance of transferred DNA makes the bacterium a suitable vector for plant genetic engineering. DNA sequences homologous to the transferred DNA of *Agrobacterium rhizogenes* were detected in some untransformed plant species suggesting a past infection by *Agrobacterium rhizogenes* during evolution of some genera, notably *Nicotiana*.

**Keywords.** *Agrobacterium rhizogenes*; Ri-plasmid; endogenous T-DNA.

## Introduction

*Agrobacterium rhizogenes* is a gram negative soil bacterium. It incites hairy root disease of many dicotyledonous plants (Brown, 1929; DeCleene and DeLey, 1981; Riker *et al.*, 1930; Siegler, 1928). The ability of *A. rhizogenes* to incite hairy root disease is determined by a virulence plasmid (Chilton *et al.*, 1982; Moore *et al.*, 1979; White and Nester, 1980) similar to that found in *Agrobacterium tumefaciens* which causes Crown gall tumors of plants. The virulence plasmid of *A. rhizogenes* is known as the Ri-plasmid to distinguish it from the tumor-inducing (Ti) plasmid. Extensive literature is available pertaining to the Ti-plasmid. Therefore, we will not describe the Ti-plasmid in detail except where necessary for comparison with the Ri-plasmids. For more information on the Ti-plasmid readers are referred to some recent review articles (Nester *et al.*, 1984; Hille *et al.*, 1984; Zambryski *et al.*, 1983).

The Ri-plasmid shares extensive functional homology with the Ti-plasmid. The Ri-plasmid, like the latter, contains a distinct segment(s) of DNA which is transferred to plant genome during infection (Chilton *et al.*, 1982; White *et al.*, 1982; Willmitzer *et al.*, 1982). The transfer of the DNA (T-DNA) to the plant genome is mediated by another segment on the plasmid known as the virulence (*vir*) region. The T-DNA confers on the plant cells the ability to grow in the absence of exogenous plant hormones. The T-DNA also confers on the transformed tissue the ability to produce modified amino acids (opines), which, in turn, are utilized only by the inciting bacteria as the carbon, nitrogen and energy source. The *Agrobacterium* species thus establish a unique ecological niche by genetically engineering the host plant—a highly sophisticated parasitism!

DNA sequences homologous to the T-DNA of Ri-plasmids were reported in some untransformed plant species (Spano *et al.*, 1982; White *et al.*, 1982, 1983; Tepfer, 1984). The presence of these homologous sequences suggest the possibility that such

Large plasmids were shown to be present in strains of *A. rhizogenes* (Schell *et al.*, 1976; Currier and Nester, 1976; White and Nester, 1980a). These strains are known to produce at least two classes of opines. One such class is represented by opines of agropine group, and the other class being the agrocinopine group. All strains of *A. rhizogenes* are known to produce agrocinopine and all or a few opines of the agropine group. The strains which produce all the agropine-type opines (agropine, mannopine, agropinic acid and mannopinic acid) are known as the agropine-type strains, whereas the strains which produce all agropine-type opines excluding agropine are known as the mannopine-type strains (Petit *et al.*, 1983; Tempé *et al.*, 1984; Tepfer and Tempé, 1981; White *et al.*, 1982; Willmitzer *et al.*, 1982).

Two T-DNA regions have been identified in agropine Ri-plasmids (see figure 1). The two tDNAs are separated from each other by about 15 Kb of non-transferred DNA. The right T-DNA ( $T_R$ ) contains genes homologous to the T-DNA from Ti-plasmids (Riseuleo *et al.*, 1982; Willmitzer *et al.*, 1982; Huffman *et al.*, 1984; Jouanin, 1984). Most important among these are the genes homologous to the *tms1* and *tms2* of the Ti-plasmid. These genes are involved in auxin biosynthesis in *A. tumefaciens* (Inze *et al.*, 1984; Schröder *et al.*, 1984; Thomashow *et al.*, 1984; Thomashow *et al.*,

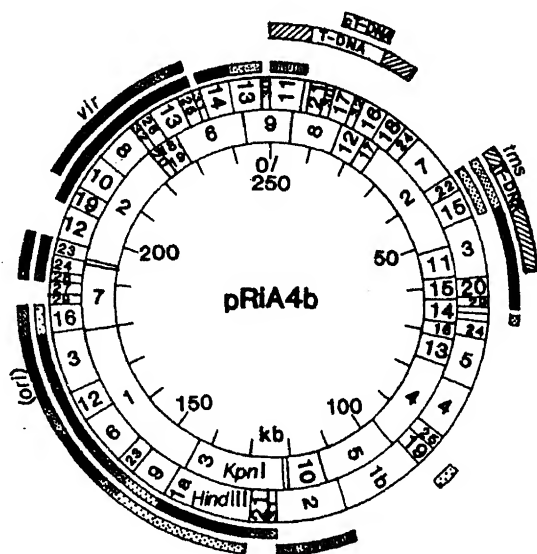


Figure 1. Restriction map of an agropine type Ri-plasmid (pRiA4b). Data taken from Huffman *et al.* (1984). Homology to the octopine type Ti-plasmid (pTiA6) is indicated by outer most thin bars while homology to the nopaline type Ti-plasmid (pTiT37) is indicated by the inner bars. Dark regions represent stronger hybridization signal.

Vir, virulence region; tms, auxin genes; ori, origin of replication; cT DNA, region of DNA homology between pRiA4b and untransformed *N. glauca*.

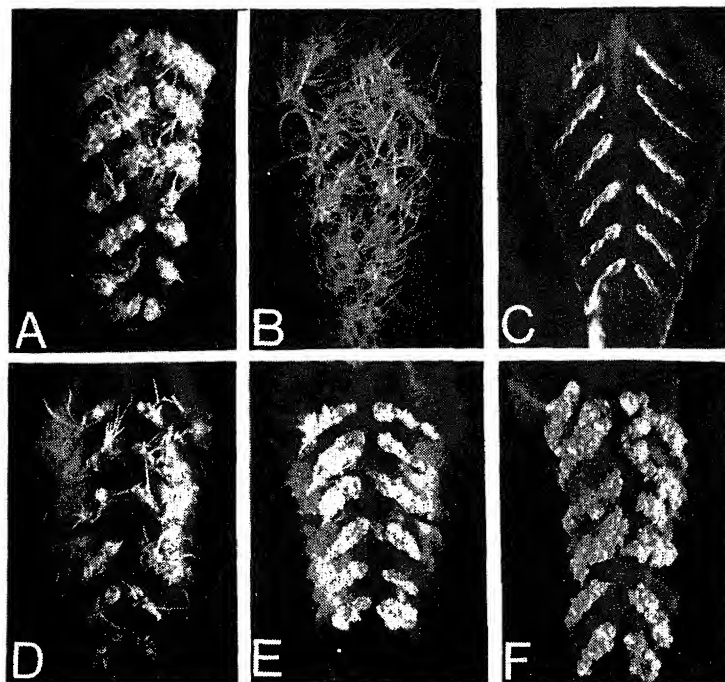
1986). Mutagenesis of this region in the Ri-plasmid is shown to result in the loss or attenuation of virulence (White *et al.*, 1985). Hybridization experiments also suggested the presence of the genes involved in agropine biosynthesis (*ags*) in the T<sub>R</sub>-DNA region although the exact number of genes involved in agropine biosynthesis is not known yet (Huffman *et al.*, 1984; Lahners *et al.*, 1984; Willmitzer *et al.*, 1982).

*Nicotiana glauca* tissues transformed with *A. rhizogenes* contain discrete m-RNA species derived from the T<sub>R</sub>-DNA. The transcripts homologous to the Ri *tms* loci in such tissues were found to be of size similar to the transcripts derived from the *tms* region of Ti-plasmids (Taylor *et al.*, 1985a; Willmitzer *et al.*, 1983). Additional transcripts were also detected from such tissues and probably correspond to the *ags* region. Similar transcripts have also been reported from carrot plants regenerated from tissues infected with *A. rhizogenes* (De Paolis *et al.*, 1985). However, the T<sub>R</sub>-DNA transcripts are not detectable in regenerated *N. glauca* plants and are either absent or present at very low levels in regenerated *Nicotiana tabacum* plants (Taylor *et al.*, 1985b; Durand-Tardif *et al.*, 1985).

The left T-DNA(T<sub>L</sub>) of agropine Ri-plasmid A4b is about 20 Kb in length but, unlike the T<sub>R</sub>-DNA does not appear to be closely related to any other characterized Ti-plasmid (Huffman *et al.*, 1984). Limited homology has been reported to the T-DNA of nopaline type Ti-plasmids, presumably to the region involved in the synthesis of agrocinopine (Huffman *et al.*, 1984; Willmitzer *et al.*, 1982). Transposon mutagenesis of the T<sub>L</sub>-DNA has revealed the presence of at least four loci (*rolA*, B, C and D) which affect tumorigenesis in some plants (White *et al.*, 1985; Estramareix *et al.*, 1986). Mutations in *rolA* (*rolA*<sup>-</sup>) results in the formation of long, straight roots giving the tumor a less compact appearance on *Kalanchoë diagraphmontiana* leaves (figure 2). The *rolB* mutation eliminates both callus or root formation at the wound site, while *rolC*<sup>-</sup> and *rolD*<sup>-</sup> mutations are more subtle (figure 2); *rolC*<sup>-</sup> resulting in retardation of root growth and *rolD*<sup>-</sup> in accentuation of callus growth giving rise to tumors resembling the Ti-plasmid infection. Transposon insertions between *rolB* and *rolC* showed weakened response on *K. diagraphmontiana* leaves implying the presence of another genetic locus (see figure 2D; White *et al.*, 1985). However, the degree of the weakened response varied considerably among the mutants in this region and between inoculations. Since such results are possible if the insertions have affected expression of the adjacent loci, no *rol* locus has been assigned to this region.

The *rol* mutations present a rather intriguing picture of the T<sub>L</sub>-DNA functions. White *et al.* (1985) observed apparent similarity between *tmr* and *rolB*<sup>-</sup> mutations on *K. diagraphmontiana* leaves. Both mutants are unable to produce any callus or roots at the infection site on the leaves. Virulence of *tmr* mutants can be restored on the leaf by complementation with the Ri T<sub>L</sub>-DNA (White *et al.*, 1985). These observations suggest that the T<sub>L</sub>-DNA is either involved in cytokinin biosynthesis or in altering cytokinin metabolism in plants. Current work in our laboratory indicates that the individual *rol* genes, including *rolB* *in trans*, are unable to restore complete virulence of *tmr* mutant strains on *Kalanchoë* leaf, thus indicating that more than one gene is involved in the synthesis or alteration of metabolism of cytokinin.

Slightom *et al.* (1986) sequenced 21,126 base-pairs of the T<sub>L</sub>-DNA and found potential for 18 open reading frames. 27 consensus border recognition sequences homologous to those found in the Ti-plasmids which determine boundaries of the T-



**Figure 2.** Effect of mutations of the  $T_L$ -DNA on tumor formation on *Kalanchoë* leaf. (A), R1000 (wild type); (B), R1022 (*rolA*<sup>-</sup>); (C), R1023 (*rolB*<sup>-</sup>); (D), R1020 (no *rol* locus assigned); (E), R1016 (*rolC*<sup>-</sup>); (F), R1244 (*rolD*<sup>-</sup>).

Pictures taken one month after infection (White *et al.*, 1985).

*al.*, 1982; Zambryski *et al.*, 1982). The variability found in the boundaries of the  $T_L$ -DNA in different plants may occur due to the preferential use of different border recognition sequences during T-DNA transfer (Slightom *et al.*, 1985; Tepfer, 1984; Taylor *et al.*, 1985a; White *et al.*, 1985). Durand-Tardif *et al.* (1985) and Taylor *et al.* (1985a) showed that the  $T_L$ -DNA is transcribed in both transformed tissue and regenerated plants. Although there are some differences in the two sets of data and all the individual transcripts are not found in every tissue or plant, careful inspection suggests that the transcripts roughly map to the open reading frames. Some of the anomalies could be explained due to turn-off and tissue-specific expression of some of the  $T_L$ -DNA genes.

Comparatively less data are available for the mannopine type Ri-plasmids. The best characterized mannopine type plasmid, that from strain 8196 contains only one T-DNA (Byrne *et al.*, 1983). Hybridization data with agropine type plasmid (pRi1855) and of pRi 8196 suggest that the Ri  $T_L$ -DNA including the *rol* loci are probably conserved (Spano *et al.*, 1982). Since the genes for mannopine synthesis are located towards the right border of the T-DNA a possible fusion of the left and right T-DNA to obtain a single T-DNA in this strain is indicated (Lahners *et al.*, 1984). The most striking difference between the agropine and mannopine type Ri-plasmids,

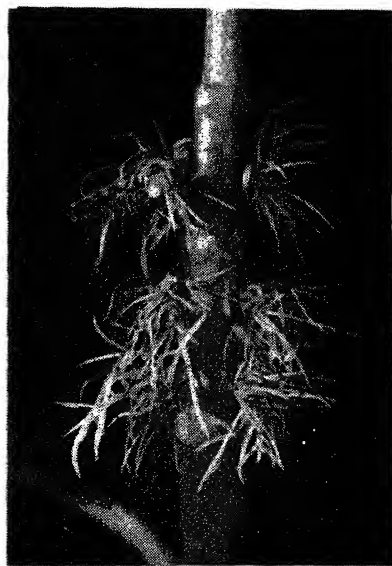
1980b; Hooykaas *et al.*, 1984; Hoekema *et al.*, 1984; Huffman *et al.*, 1984; Willmitzer *et al.*, 1982). Hybridization data between pRiA4b and the two most studied Ti-plasmids, pTiA6 and pTiT37, suggest that all the known *vir* loci are present on the Ri-plasmid (Huffman *et al.*, 1984; Unger *et al.*, 1985). One exception to this finding is the lack of hybridization to *virE* probe. *Vir* mutations in Ti-plasmid including *virE* can, however, be complemented by the virulence region of the Ri-plasmid (Hooykaas *et al.*, 1984; White, F.F., unpublished results). The reason for the lack of the homology to the *virE* despite of functional complementation is yet unknown.

### *Ri-transformed plants*

*A. rhizogenes* induced tumors have a distinct morphological phenotype on many plant species, notably the pronounced rooting, compared to tumors derived from strains containing Ti-plasmids (figure 3). Moreover, the Ri-plasmid transformation of plants does not appear to be inhibitory to normal differentiation as the incorporation of T-DNA of the Ti-plasmid containing the phytohormone synthesis genes. Plants can be readily regenerated from the callus or roots of Ri-transformed plant tissues (Ackermann, 1977; Chilton *et al.*, 1982; David *et al.*, 1984; Spano and Costantino, 1982; Tepfer, 1982, 1984; Taylor *et al.*, 1985a), and the T-DNA can be



A



B

**Figure 3.** (A), Tumor formation by *A. tumefaciens*, A348 (octopine type plasmid), on *Helianthus* sp. (B), Root proliferation induced by *A. rhizogenes* A4b on the stem of *K. diagamontiana*.



subsequently transmitted to progeny plants (Costantino *et al.*, 1984; Tepfer, 1984; Taylor *et al.*, 1985a; Sinkar V. P., unpublished results). The analysis of T-DNA of the regenerated plants suggest that the incorporation of the *tms* genes is inhibitory to regeneration in some species. In such species, *e.g.*, *N. glauca*, only those cells which either have lost the *tms* genes or the T<sub>R</sub>-DNA or never received the T<sub>R</sub>-DNA regenerate whole plants (Taylor *et al.*, 1985b). On the other hand, the regeneration process in some plants such as *N. tabacum* appears to be less affected by the presence of the *tms* genes (Taylor *et al.*, 1985a). In some instances, however, regenerated *N. tabacum* plants do not contain any T<sub>R</sub>-DNA (Durand-Tardif *et al.*, 1985; Sinkar V. P., unpublished results). As a general rule, it seems that severely reduced activity or inactivation of the *tms* loci is a prerequisite for plant regeneration from the Ri-plasmid transformed tissue.

Regenerated plants containing Ri-plasmid T-DNA often exhibit many phenotypic abnormalities, such as wrinkled leaves, shortened internode distances, loss of apical dominance, increased lateral root-formation and heterostyly (figure 4); Ackermann, 1977; Tepfer, 1984; Taylor *et al.*, 1985b; Ooms *et al.*, 1985; Sinkar V. P., unpublished results). In wrinkled *N. glauca*, the T<sub>R</sub>-DNA pattern indicates that complete copies of either the *tms* loci or entire T<sub>R</sub>-DNA sequences were absent (Taylor *et al.*, 1985b; Sinkar V. P., unpublished results). Similar observations have also been made with *N. tabacum* and *Solanum tuberosum* (Durand-Tardif *et al.*, 1985; Sinkar V. P., unpublished results; Ooms *et al.*, 1985). However, all abnormal plants tested so far always showed the presence of the T<sub>L</sub>-DNA. Thus, it seems that some gene or combination of genes in the T<sub>L</sub>-DNA controls the aberrant phenotype associated with the regenerated plants.



Initial characterization of T-DNA both with agropine and mannopine Ri-plasmids led to the observation of hybridization to some untransformed plant species. In the case of the agropine type Ri-plasmid, A4b, strong hybridization to *N. glauca* genome was observed with the T<sub>L</sub>-DNA of the plasmid (White *et al.*, 1982; White *et al.*, 1983). The homologous plant sequences (about 12 Kb in size) were cloned and sequenced (Furner *et al.*, 1986). Of these 12 Kb, about 7.5–8.0 Kb is the central T-DNA region and the remainder is in asymmetric inverted repeats (figure 5). Similar homologous sequences were also detected in other species of *Nicotiana*, e.g., *N. tabacum*, *N. octophora*, *N. tomentosiformis* and *N. benavidesii* (Furner *et al.*, 1986). Various wild isolates of *N. glauca* obtained from different parts of the world also showed the presence of similar sequences, although restriction patterns differed (Furner *et al.*, 1986).

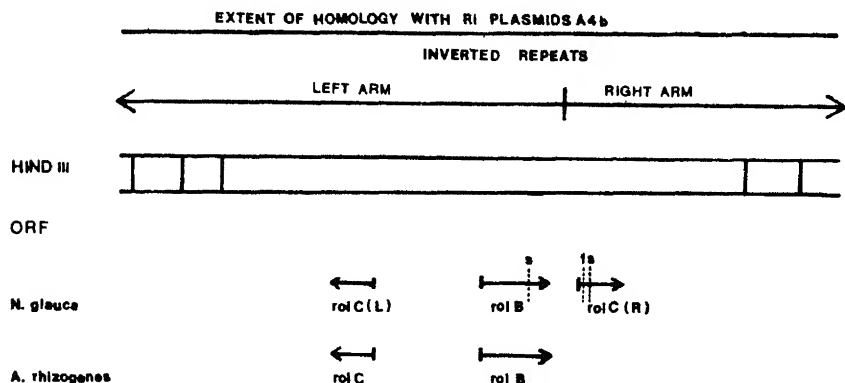


Figure 5. Organization of Ri T<sub>L</sub>-DNA homologous sequence *N. glauca*. Hind III map of the homologous region is shown. Open reading frames deduced from DNA sequence data are depicted as arrows at the bottom. Corresponding open reading frames from the Ri-plasmid A4b are shown for comparison. Beginning of the inverted repeats is represented by a vertical bar. (Data taken from Furner *et al.*, 1986).

(ORF), Open reading frame; (F), frameshift; (S), stop codon; (L), left; (R), right.

The region of homology between *N. glauca* and pRiA4b includes *rolC*, *rolB* and an undetermined region of *rolD*. DNA sequence analysis showed that the left plant copy of the *rolC* locus contains the complete open reading frame. The right copy of the plant *rolC* contains a frame shift and a termination codon near the start codon implying that this copy is not potentially functional. The open reading frame of the plant *rolB* can potentially encode a polypeptide that is truncated by 48 amino acids at the carboxyl terminus (see figure 5, Furner *et al.*, 1986). These open reading frames share about 75% DNA and 84% amino acid homology with the corresponding regions of the Ri-plasmid.

Many questions are raised by the presence of endogenous T-DNA. The foremost among them are whether the endogenous T-DNA contains any active genes and, if

genes are either silent or are expressed only transiently. Experiments are in progress to use the plant genes to complement Ri-plasmid mutations in *rolB* and *rolC*. These experiments including analysis of methylation pattern and DNase sensitivity of the plant chromatin containing sequences homologous to the T-DNA may provide answers to some of the pertinent questions.

Many plant species do not contain these homologous sequences. Moreover, the amount of homologous DNA in various species differs greatly (Furner *et al.*, 1986). These facts support the hypothesis that the genes may simply represent a genetic scar and may be eventually eliminated from the plant genome. However, the fact that only a single frame shift has occurred in 1957 base-pairs of open reading frame compared to 25 frameshifts in 1240 base-pairs of intergenic region in *N. glauca* compared to present day *A. rhizogenes* indicates that selection may have maintained open reading frames in both the organisms.

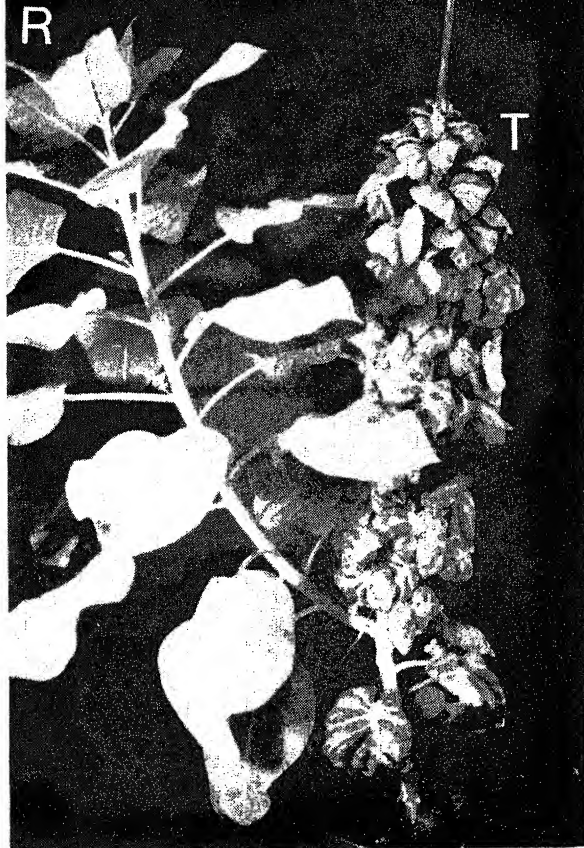
Plants regenerated from tissues transformed with *A. rhizogenes* often show an abnormal phenotype. This phenotype is associated with the presence and expression of T<sub>L</sub>-DNA in the plants (Tepfer, 1984; Taylor *et al.*, 1985b). Such plants frequently revert to a normal phenotype (figure 6). Transcriptional inactivation of the T-DNA is responsible for the phenomenon of reversion (Durand-Tardif *et al.*, 1985; Sinkar V. K., unpublished results). The revertant shoots from abnormal plants show very high growth rate and out compete the abnormal shoots. In nature while competing for light and nutrients the revertant shoot would have an advantage. Moreover, the revertant shoots are able to set higher number of viable seeds, and the F<sub>1</sub> progeny plants have normal appearance. These observations can at least partly explain the occurrence of the T-DNA homologous sequences in normal plants.

Acquisition of bacterial genes by higher plants may possibly represent as significant mode of evolution. Analysis of plants for homologous sequences using various other T-DNA probes will help to clear the picture. So far, only a few T-DNAs have been carefully analyzed and fewer have been used for homology study. A few instances of homology between the Ti-plasmid and *N. tabacum* DNA have been reported (Thomashow *et al.*, 1980; Yang and Simpson, 1981). These observations have not been pursued further.

## Conclusions

While Ri- and Ti-plasmids are clearly related structurally and functionally, and they appear to have evolved from a common ancestor, they have diverged significantly and should be considered separate. Large segments of pRiA4b show little or no homology to the Ti-plasmid. Particularly notable is the absence of homology of the pRiA4b T<sub>L</sub>-DNA to any known Ti-plasmids (Huffman *et al.*, 1984).

The Ri T-DNA region can be used for the construction of plant transformation vectors similar to the Ti-plasmid derived vectors (An *et al.*, 1985; Klee *et al.*, 1985; Zambryski *et al.*, 1983). Since the T<sub>L</sub>-DNA confers growth abnormalities on plants; deletion of morphogenic loci from the T<sub>L</sub>-DNA is essential for general use of the



**Figure 6.** Reversion of transformed *N. tabacum*. *N. glauca* plant regenerated from leaf tissue transformed with *A. rhizogenes*. Note normal appearing revertant shoots (R) emerging from the abnormal (T).

by *Rhizobium meliloti* strains which contain Ri-plasmid. Another interesting application of the bacterium was found in the development of drought-resistance (Strobel and Nachmias, 1985). Application of *A. rhizogenes* to bare root stock of almond trees resulted in a striking increase in root mass after 90 days. Moreover, increases in number of roots, leaves, stem diameter and shoot elongation were seen during the first growing season after the treatment. No pathological reaction was seen with any of the plants used in the study. This approach, therefore, may prove to be useful while transferring drought-sensitive plants from the nursery to the field.

Perhaps the most important contribution of the Ri-plasmid is that it provided impetus to the study of genetic exchange between plants and bacteria and initiated studies on the effects of the exchange of genetic information on evolution of both the groups. At the moment, the Ri-plasmid-plant exchange seems to be the only known system where such an exchange has occurred between the two kingdoms. Genetic exchange between other eukaryotes and bacteria has, however, been postulated in two other instances. It appears that a symbiotic bacterium *Photobacterium leiognathi*

Fridovich, 1981). Similarly, microorganisms isolated from human urine have been reported to produce a material immunologically related to human chorionic gonadotropin (Cohen and Strampp, 1976). *A. rhizogenes* differs from these bacteria at least in one respect, i.e., it does not share any special relationship with the genus *Nicotiana*. In fact, the natural strains of *A. rhizogenes* were isolated from apple or rose (Hedgecock, 1905; Riker *et al.*, 1930; Munneche *et al.*, 1963). Hence, the occurrence of T-DNA homologous sequences in *Nicotiana* species presents a unique case. It is possible that such sequences are present in other plants but have diverged to an extent that they are impossible to detect either with pRiA4b or *Nicotiana glauca* probes.

## Acknowledgements

The authors thank Drs. Srinivasan and T. Parsons for critical reading of the manuscript and helpful suggestions. This work has been supported by funds from the American Cancer Society, the National Science Foundation, the United States Department of Agriculture, and Philip Morris, Inc., USA.

## References

- Ackermann, C. (1977) *Plant Sci. Lett.*, **8**, 23.
- An, G., Watson, B. D., Stachel, S., Gordon, M. P. and Nester, E. W. (1985) *EMBO J.*, **4**, 277.
- Brown, N. A. (1929) *J. Agric. Res. (Washington DC)*, **39**, 747.
- Byrne, M. C., Koplow, J., David, C., Tempé, J. and Chilton, M.-D. (1983) *J. Mol. Appl. Genet.*, **2**, 201.
- Chilton, M.-D., Tepfer, D. A., Petit, A., David, C., Casse-Delbart, F. and Tempé, J. (1982) *Nature (London)*, **295**, 432.
- Comai, L., Facciotti, D., Hiatt, W. R., Thompson, G., Rose, R. E. and Stalker, D. M. (1985) *Nature (London)*, **317**, 741.
- Cohen, H. and Strampp, A. (1976) *Proc. Soc. Exp. Biol. Med.*, **152**, 408.
- Costantino, P., Spano, L., Pomponi, M., Benvenuto, E. and Ancora, G. (1984) *J. Mol. Appl. Genet.*, **2**, 465.
- Currier, T. C. and Nester, E. W. (1976) *J. Bacteriol.*, **126**, 157.
- David, C., Chilton, M.-D. and Tempe, J. (1984) *Biotechnology*, **2**, 73.
- De Cleene, M. D. and De Ley, J. (1981) *Bot. Rev.*, **47**, 147.
- DePaolis, A., Mauro, M. L., Pomponi, M., Cardarelli, M., Spano, L. and Costantino, P. (1985) *Plasmid*, **13**, 1.
- Durand-Tardif, M., Broglio, R., Slightom, J. and Tepfer, D. (1985) *J. Mol. Biol.*, **186**, 557.
- Estramareix, C., Ratet, P., Boulanger, F. and Richaud, F. (1986) *Plasmid*, **15**, 245.
- Furner, I. J., Huffman, G. A., Amasino, R. M., Garfinkel, D. J., Gordon, M. P. and Nester, E. W. (1986) *Nature (London)*, **329**, 422.
- Hedgecock, G. G. (1905) *U. S. Dept. Agric. Bur. Plant Ind. Bull.*, **131**, 21.
- Hille, J., Hoekema, A., Hooykaas, P. and Schilperoort, R. (1984) in *Plant Gene Research* (eds D. P. S. Verma and Th. Hohn) (Springer-Verlag/Wien.) p. 271.
- Hoekema, A., Hooykaas, P. J. and Schilperoort, R. A. (1984) *J. Bacteriol.*, **158**, 383.
- Hooykaas, P. J. J., Hofker, M., den Dulk-Ras, H. and Schilperoort, R. A. (1984) *Plasmid*, **11**, 195.
- Huffman, G. A., White, F. F., Gordon, M. P. and Nester, E. W. (1984) *J. Bacteriol.*, **157**, 269.
- Inze, D., Follin, A., Van Lijsebettens, M., Simoens, C., Genetello, C., Van Montagu, M. and Schell, J. (1984) *Mol. Gen. Genet.*, **194**, 265.
- Jensen, J. S., Marcker, K. A., Otten, L. and Schell, J. (1986) *Nature (London)*, **321**, 669.
- Jouanin, L. (1984) *Plasmid*, **12**, 91.
- Klee, H. J., Yanofsky, M. F. and Nester, E. W. (1985) *Biotechnology*, **3**, 637.
- Lahners, K., Byrne, M. C. and Chilton, M. (1984) *Plasmid*, **11**, 130.
- Martin, J. P., Jr. and Fridovich, I. (1981) *J. Biol. Chem.*, **256**, 6080.
- Moore, J. W., Gordon, M. P. and Stachel, G. (1979) *Plasmid*, **2**, 617.

- Nester, E. W., Gordon, M. P., Amasino, R. M. and Yanofsky, M. F. (1984) *Annu. Rev. Plant Physiol.*, **35**, 387.
- Ooms, G., Karp, A., Burrell, M. M., Twell, D. and Roberts, J. (1985) *Theor. Appl. Genet.*, **70**, 440.
- Petit, A., David, C., Dahl, G., Ellis, J., Guyon, P., Casse-Delbart, F. and Tempé, J. (1983) *Mol. Gen. Genet.*, **190**, 204.
- Riker, A. J., Banfield, W. M., Wright, W. H., Keitt, G. W. and Sagen, H. E. (1930) *J. Agric. Res.*, **41**, 507.
- Riseuleo, G., Battistoni, P. and Costantino, P. (1982) *Plasmid*, **7**, 45.
- Schell, J., Van Montagu, M., Depicker, A., DeWaele, D., Engler, G., Genetello, C., Hernalsteens, J. O., Holsters, M., Messens, E., Silva, B., Van den Elsacken, S., Van Larabeke, N. and Zaenen, I. (1976) in *Nucleic Acids and Protein Synthesis in Plants* (eds L. Bogorad and J. H. Weil) (New York: Plenum Press) p. 329.
- Schröder, G., Waffenschmidt, S., Weiler, E. W. and Schröder, J. (1984) *Eur. J. Biochem.*, **138**, 387.
- Siegler, E. A. (1928) *J. Agric. Res. (Washington DC)*, **37**, 301.
- Simpson, R. B., O'Hara, P. J., Kwok, J., Montoya, A. L., Lichtenstein, C., Gordon, M. P. and Nester, E. W. (1982) *Cell*, **29**, 1005.
- Slightom, J. L., Durand-Tardif, M., Jouanin, L. and Tepfer, D. (1986) *J. Biol. Chem.*, **261**, 108.
- Slightom, J. L., Jouanin, L., Leach, F., Drong, R. F. and Tepfer, D. (1985) *EMBO J.*, **4**, 3069.
- Spano, L. and Costantino, P. (1982) *Z. Pflanzenphysiol.*, **106**, 87.
- Spano, L., Pomponi, M., Costantino, P., Van Slogteren, G. M. S. and Tempé, J. (1982) *Plant Mol. Biol.*, **1**, 291.
- Strobel, G. A., Lam, B., Harrison, L., Hess, B. and Lam, S. (1985) *J. Gen. Microbiol.*, **131**, 355.
- Strobel, G. A. and Nachmias, A. (1985) *J. Gen. Microbiol.*, **131**, 1245.
- Taylor, B. H., Amasino, R. M., White, F. F., Nester, E. W. and Gordon, M. P. (1985a) *Mol. Gen. Genet.*, **201**, 554.
- Taylor, B. H., White, F. F., Nester, E. W. and Gordon, M. P. (1985b) *Mol. Gen. Genet.*, **201**, 546.
- Tempé, J., Petit, A. and Farrand, S. K. (1984) in *Plant Gene Research* (eds D. P. S. Verma and Th. Hohn) (Springer-Verlag/Wien.) p. 271.
- Tepfer, D. (1982) *2<sup>e</sup> Colloque sur les Recherches Fruitieres-Bordeaux*: 47-59.
- Tepfer, D. (1984) *Cell*, **37**, 959.
- Tepfer, D. A. and Tempé, J. (1981) *CR Acad. Sci. Paris, Ser III*, **292**, 153.
- Thomashow, L. S., Reeves, S. and Thomashow, M. F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5071.
- Thomashow, M. F., Hugly, S., Buchholtz, W. G. and Thomashow, L. S. (1986) *Science*, **232**, 606.
- Thomashow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P. and Nester, E. W. (1980) *Cell*, **19**, 729.
- Unger, L., Ziegler, S. F., Huffman, G. A., Knauf, V. C., Peet, R., Moore, L. W., Gordon, M. P. and Nester, E. W. (1985) *J. Bacteriol.*, **164**, 723.
- White, F. F., Garfinkel, D. J., Huffman, G. A., Gordon, M. P. and Nester, E. W. (1983) *Nature (London)*, **301**, 348.
- White, F. F., Ghidossi, G., Gordon, M. P. and Nester, E. W. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3193.
- White, F. F. and Nester, E. W. (1980a) *J. Bacteriol.*, **141**, 1134.
- White, F. F. and Nester, E. W. (1980b) *J. Bacteriol.*, **144**, 710.
- White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P. and Nester, E. W. (1985) *J. Bacteriol.*, **164**, 33.
- Willmitzer, L., Dhaese, P., Schreier, P. H., Schmalenbach, W., Van Montagu, M. and Schell, J. (1983) *Cell*, **32**, 1045.
- Willmitzer, L., Sanchez-Serrano, J., Busfeld, E. and Schell, J. (1982) *Mol. Gen. Genet.*, **186**, 16.
- Yadav, N. S., Vanderleyden, J., Bennett, D. R., Barnes, W. M. and Chilton, M.-D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6322.
- Yang, F. and Simpson, R. B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4151.
- Zambryski, P., Depicker, A., Kruger, D. and Goodman, H. M. (1982) *Mol. Gen. Genet.*, **1**, 361.
- Zambryski, P., Goodman, H., Van Montagu, M. and Schell, J. (1983) in *Mobile Genetic Elements* (ed. J. Shapiro) (Academic Press) p. 506.
- Zambryski, P., Joos, H., Gentello, C., Leemans, J., Van Montagu, M. and Schell, J. (1983) *EMBO J.*, **2**, 2143.



# Mode of action of lipoic acid in diabetes

S. S. WAGH, C. V. NATRAJ and K. K. G. MENON

Hindustan Lever Research Centre, Andheri (East), Bombay 400 099, India

**Abstract.** Metabolic aberrations in diabetes such as hyperglycemia, ketonemia, ketonuria, reduced glycogen in tissues and reduced rates of fatty acid synthesis in the liver are corrected by the administration of lipoic acid. Dithiol octanoic acid is formed from lipoic acid by reduction and substitutes for Coenzyme A in several enzymatic reactions such as pyruvate dehydrogenase, citrate synthase, acetyl Coenzyme A carboxylase, fatty acid synthetase, and triglyceride and phospholipid biosynthesis; but not in the oxidation of fatty acids because of the slow rates of thiolysis of  $\beta$ -keto acyl dithiioctanoic acid. The overall effect of these changes in the key enzymic activities is seen in the increased rates of oxidation of glucose and a reduction in fatty acid oxidation in diabetes following lipoic acid administration.

**Keywords.** Lipoic acid; diabetes; Coenzyme A; pyruvate dehydrogenase; lipid biosynthesis; acetoacetate metabolism.

## Introduction

Diabetes is a disease that affects a significant proportion of the population and is characterised by abnormal carbohydrate, fat and protein metabolisms. There are two types of diabetes: type I (juvenile diabetes) and type II (maturity onset diabetes). Type I diabetes, is characterised by low levels of serum insulin and these patients have an absolute requirement for insulin. On the other hand, type II diabetics have normal or supranormal levels of serum insulin and are often obese (Chiles and Tzagournis, 1970). The causes of type I diabetes are many including genetic predisposition (Foster, 1983), overproduction of counter regulatory hormones such as glucagon, growth hormone, etc. (Zadik *et al.*, 1980) presence of insulinase and insulin antibodies (Neufeld *et al.*, 1980). The causes of type II diabetes have been attributed to a reduction in hormone receptor levels on target cells and impairment of 'post-receptor' functions (Olefsky and Kotterman, 1981).

The major biochemical abnormalities in diabetes are increased blood sugar (hyperglycemia) decreased glucose tolerance, urinary sugar (glucosuria), increased serum pyruvate, lactate, acetoacetate and triglycerides, reduced glycogen in the tissues, impaired fat biosynthesis in the liver and increased gluconeogenesis in the liver and kidney.

In earlier studies from this laboratory, we have shown that lipoic acid or dithiioctanoic acid (DTO) administration in alloxan diabetic rats brings about significant reductions in blood sugar, serum pyruvate and acetoacetate levels and increases in liver glycogen and fat synthesis (Natraj *et al.*, 1984; Gandhi *et al.*, 1985). Further, relative  $\alpha$ -lipoic acid content of alloxan diabetic rat livers was considerably less than



normalising increased serum pyruvate and impaired liver pyruvate dehydrogenase (EC 1.2.4.1) characteristic of the diabetic state (Gandhi *et al.*, 1985).

The metabolic disposition of pyruvate occurs by 3 pathways: (i) production of lactate through lactate dehydrogenase (EC 1.1.1.27)—lactic acidemia is a characteristic of uncontrolled diabetes; (ii) conversion of pyruvate by carboxylation to oxalacetate—pyruvate carboxylation is increased in diabetes in an effort to produce glucose by gluconeogenesis and (iii) decarboxylation to acetyl Coenzyme A (CoA) and CO<sub>2</sub>. High production of acetyl CoA through fatty acid oxidation is known to inhibit pyruvate dehydrogenase and also to stimulate pyruvate carboxylase (EC 6.4.1.1). We found that acetyl DTO does not stimulate pyruvate carboxylase and DTO in fact activates pyruvate dehydrogenase.

Whereas DTO can substitute for CoA in a variety of enzymic reactions (*vide infra*),  $\beta$ -keto fatty acyl lipoate is sparsely thiolized to yield acetyl dihydrolipoic acid (acetyl DTO). The reduced thiolysis of  $\beta$ -keto fatty acyl DTO and the lack of activation of pyruvate carboxylase by acetyl DTO could account for the antiketotic and anti-gluconeogenic effects of lipoic acid. The metabolic role of lipoic acid as evidenced by its participation in a number of enzymatic reactions involved in carbohydrate and lipid metabolism will be discussed in this paper with a view to focus attention on the significance of DTO in general metabolism as well as its specific role in the treatment of diabetes.

### *Pyruvate dehydrogenase*

Lipoic acid is a cofactor in the multienzyme complexes of  $\alpha$ -keto acid dehydrogenases such as pyruvate,  $\alpha$ -keto glutarate and branched chain  $\alpha$ -keto acid dehydrogenases. Pyruvate is a metabolite located at the nexus of the 3 metabolisms affected in diabetes, *viz.*, carbohydrate, fat and protein metabolisms. We had shown earlier that lipoic acid levels are reduced in diabetes and administration of lipoic acid stimulates pyruvate dehydrogenase (Gandhi *et al.*, 1985). It is probable therefore that some of the effects of lipoic acid such as reduction in serum pyruvate and part of the blood sugar reduction could be explained through increased rates of utilization of pyruvate and therefore glucose for energy generation and for anabolic reactions. In this connection, it must be mentioned that administration of dichloroacetate which also stimulates pyruvate dehydrogenase, lowers serum pyruvate and blood sugar in diabetic rats (Whitehouse and Randle, 1973).

Although dichloroacetate and lipoic acid stimulate pyruvate dehydrogenase and bring about reductions in serum pyruvate and blood sugar levels, they differ in at least one crucial respect, *i.e.*, while lipoic acid brings down serum acetoacetate levels (Natraj *et al.*, 1984) dichloroacetate administration does not (Stackpoole *et al.*, 1978; Gerard *et al.*, 1979). It is reasonable to conclude that stimulation of pyruvate dehydrogenase alone cannot explain all the observed effects of lipoic acid in diabetes and any proposal for the mode of action of lipoic acid must explain the reduction in ketosis.

The role of lipoic acid as a carrier of acetyl groups and electrons between pyruvate and CoA in the enzyme complex of pyruvate dehydrogenase has been well establi-

genase kinase and pyruvate dehydrogenase phosphatase, respectively (Kerboey *et al.*, 1976). In streptozotocin diabetic rats, it has been shown that the fraction of the enzyme in the active form is reduced, while the total amount of the enzyme is not changed (Weinberg and Utter, 1980).

Apart of the loss in enzyme activity in diabetes could be due to the reduction in lipoic acid substitution at the enzyme level. However, this does not explain the stimulation of pyruvate dehydrogenase following lipoic acid administration in *normal rats* where lipoic acid substitution is presumed to be optimal.

In order to explain this, it is necessary to take a closer look at the effects of the product, acetyl CoA on the enzyme activity.

Wieland *et al.* (1971) have shown that in the presence of acetyl CoA, the enzyme undergoes inactivation. The mechanism of this inactivation has been shown to be through stimulation of the kinase (Cate and Roche, 1983). Dichloroacetate inhibits pyruvate dehydrogenase kinase thus stimulating pyruvate dehydrogenase.

In diabetes, as is well known, increased oxidation of fatty acids occurs. As a result, acetyl CoA accumulates in the liver mitochondria. The observed increase in the proportion of the inactive form of pyruvate dehydrogenase could be a direct consequence of this. It has also been shown that this inactivation of the enzyme can be reversed by incubating the inactive enzyme with CoA (Cate and Roche, 1983). The question is whether DTO can substitute for CoA in this reaction.

Pyruvate dehydrogenase isolated from normal rat liver mitochondria has been assayed with DTO in the complete absence of CoA, and for the first time, it has been shown that pyruvate can be converted to acetyl DTO (Gandhi *et al.*, 1985). It is therefore probable that in the diabetic liver when the enzyme is inactivated due to acylation of the enzyme bound lipoic acid, DTO could help overcome the inhibition by deacylating the enzyme. The same argument can be extended to normal rat liver pyruvate dehydrogenase where it is probable that a certain proportion of the enzyme exists in the catalytically inactive form due to acylation of enzyme bound lipoic acid.

Lipoic acid administration in diabetes therefore affects pyruvate dehydrogenase in two ways; activation by deacylating the enzyme bound acetyl groups and secondly, by reducing the rate of acetyl CoA production by inhibiting fatty acid oxidation.

## Pyruvate carboxylase

Pyruvate carboxylase catalyzes and initiates gluconeogenesis from pyruvate and alanine (Utter *et al.*, 1964). It is an allosteric enzyme and from different sources, it has been shown that it has an absolute requirement for acetyl CoA for maximum activity. The activity and the number of copies of this enzyme have been shown to be increased in streptozotocin diabetes (Weinberg and Utter, 1980). Increased gluconeogenesis contributes to the hyperglycemic condition in diabetes.

Pyruvate carboxylase has been isolated from normal rat liver mitochondria and assayed by coupling with excess citrate synthase (EC 4.1.3.7). The overall reaction is the conversion of pyruvate in the presence of acetyl CoA or acetyl DTO to oxalacetate followed by the condensation of oxalacetate with acetyl CoA or acetyl DTO to citrate. The formation of citrate was monitored by measuring the free thiol relea-

sed from acetyl CoA or acetyl DTO using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and also by estimating citrate colourimetrically. The specific activity of the enzyme with acetyl CoA as the modulator was 43.82 n mol of citrate formed/min/mg protein, whereas with acetyl DTO no citrate could be detected indicating that acetyl DTO does not stimulate oxalacetate formation from pyruvate (Gandhi *et al.*, 1985). Lipoic acid administered to diabetic animals can get converted to acetyl DTO, which does not stimulate gluconeogenesis, thus contributing to a reduction in blood sugar levels.

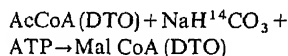
### DTO in fatty acid, triglyceride and phospholipid synthesis

We have demonstrated for the first time that DTO can participate in the acetate activation step to form acetyl DTO which can be carboxylated to malonyl DTO with subsequent formation of palmitate in the same way as CoA does in fatty acid biosynthesis (Wagh *et al.*, 1986). Acetyl CoA synthetase (EC 6.2.1.1) was isolated from rat liver mitochondria and the assay was carried out in the presence of ATP and CoA or DTO. Acetyl CoA or acetyl DTO formation was monitored by the hydroxamate assay (Jones and Lipmann, 1955). The specific activity of the enzyme with CoA was 1.61 and with DTO 0.78  $\mu$  mol of product formed/min/mg protein. DTO was approximately half as effective as CoA in this reaction.

Apart from acetate activation, DTO is also effective in the activation of fatty acids to fatty acyl DTO. Fatty acyl CoA synthetase (EC 6.2.1.3) was isolated from normal rat liver microsomes. The reaction was monitored by taking [ $^{14}$ C]-palmitic acid and incubating with CoA or DTO in the presence of enzyme and ATP. The product was extracted, separated on thin-layer chromatography (TLC) and the radioactivity incorporated into fatty acyl CoA or fatty acyl DTO was measured. The specific activity with CoA was found to be 32 and with DTO 44  $\mu$  mol DTO acylated/min/mg protein. In this reaction, DTO was found to be comparable to CoA (Gandhi *et al.*, 1985).

On the other hand, the specific activity of acetyl DTO in the carboxylation to malonyl DTO is only about 60% of that of acetyl CoA to malonyl CoA (Gandhi *et al.*, 1985) (table 1).

**Table 1.** Acetyl CoA carboxylase.



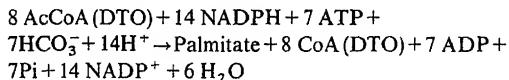
Substrate	CPM incorporated	Sp. activity*
AcCoA	4248	74.9
AcDTO	2535	44.7

\*n mol of CO<sub>2</sub> fixed/min/mg protein.

Acetyl CoA carboxylase was isolated from rat liver cytosol and partially purified. The incorporation of [ $^{14}$ C]-NaHCO<sub>3</sub> into acid stable counts was used to assay the enzyme. The product was identified as malonyl DTO.

Similarly, acetyl DTO is only about half as effective as acetyl CoA in the fatty acid

TABLE 2. Fatty acid synthetase.



Substrate	n mol NADPH oxidized/ min/mg protein	Activity
		n mol [ $^{14}\text{C}$ ]-acetyl CoA/ DTO incorporated/min/mg protein
AcCoA	75.0 (5.6)	43.2 (5.4)
AcDTO	37.0 (2.7)	22.2 (2.7)

Fatty acid synthetase was isolated from normal rat livers (Stoops *et al.*, 1979) and the activity estimated by coupling with acetyl CoA carboxylase and determining the rate of oxidation of NADPH measured spectrophotometrically or the radioactivity incorporated from [ $^{14}\text{C}$ ]-acetyl CoA or [ $^{14}\text{C}$ ]-acetyl DTO into palmitate which was isolated from the reaction mixture and purified by TLC. Numbers in parentheses are the n mol of palmitate produced/min/mg protein.

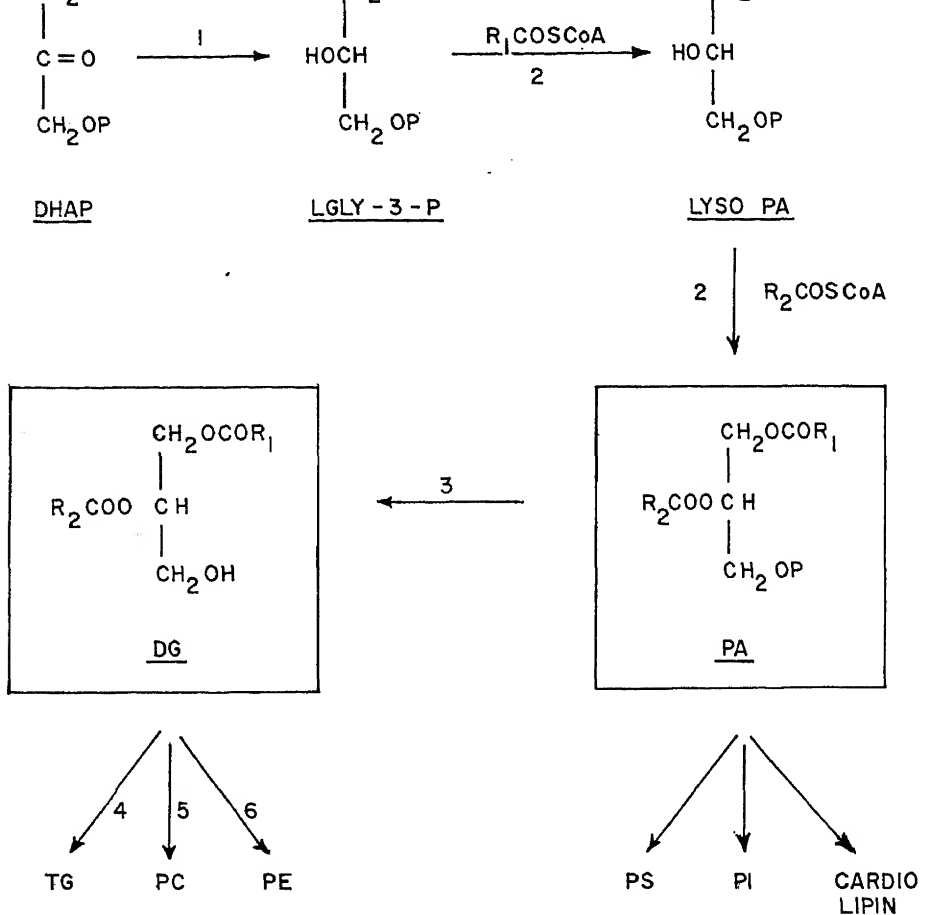
In nature, fatty acids are converted either to triglycerides or phospholipids. Figure 1 gives a skeletal view of the biosynthesis of triglycerides and some of the phospholipids. Sn-3-glycerol phosphate formed from dihydroxy acetone phosphate is usually acylated by a saturated fatty acyl CoA in the  $\alpha$ -position and subsequently by an unsaturated fatty acyl CoA in the  $\beta$ -position to yield phosphatidic acid. Phosphatidic acid is converted to phosphatidyl serine, phosphatidyl inositol or cardiolipin by a series of steps involving cytidine triphosphate. Through the action of a phosphatase, phosphatidic acid is converted to  $\alpha$ - $\beta$ -diglyceride which is the substrate for the formation of either triglycerides or phosphatidyl choline (lecithin) or phosphatidyl ethanolamine (cephalin). We have examined whether triglyceride and phospholipid biosynthesis could take place with DTO esters of saturated and unsaturated fatty acids in the same way CoA esters do using rat liver microsomes (table 3).

These experiments were conducted using [ $\text{U-}^{14}\text{C}$ ]-labelled glycerol 3 phosphate and table 3 shows that the synthesis of total lipids occurs, in fact better with palmitoyl + oleyl DTO than that with palmitoyl + oleyl CoA. This increase in lipid synthesis using fatty acyl DTO is reflected not so much in neutral lipid synthesis but more in the synthesis of phosphatidic acid and more particularly in the synthesis of phosphatidyl choline (lecithin).

### DTO, fatty acid oxidation and acetoacetate metabolism

It is now well established that ketosis occurs primarily due to increased oxidation of fats in diabetes (Ohgaku *et al.*, 1983). Therefore, the effect of lipoic acid administration on fatty acid and glucose oxidation in normal and diabetic rats was investigated.

Having shown that DTO substitutes for CoA in the biosynthesis of fatty acids, triglycerides as well as phospholipids with more or less equal facility, we were interested in examining more closely the various steps in fatty acid oxidation with a view to see whether DTO participates in fatty acid oxidation as well. As shown in



- 1 GLYCEROL 3 PHOSPHATE DEHYDROGENASE
- 2 GLYCEROL PHOSPHATE ACYL TRANSFERASE
- 3 PHOSPHATIDATE PHOSPHATASE
- 4 DIACYL GLYCEROL ACYL TRANSFERASE
- 5 PHOSPHOCHOLINE TRANSFERASE
- 6 PHOSPHOETHANOLAMINE TRANSFERASE

Figure 1. Triglyceride and phospholipid biosynthesis.

Acetoacetyl CoA can form acetoacetate either by deacylation (Segal and Menon, 1961) or by cleavage from hydroxy methyl glutaryl (HMG) CoA (Sauer and Erfle, 1966). Further, acetoacetate can also be formed from acetoacetyl CoA by the thio-phorase (EC 2:8:3:5) reaction involving succinyl, malonyl and other dicarboxylic substrates (Hersh and Jencks, 1967; Moore and Jencks, 1982). Acetoacetyl CoA is biosynthesized through the reversal of the thiolase (EC 2:3:1:0) reaction, i.e. from two

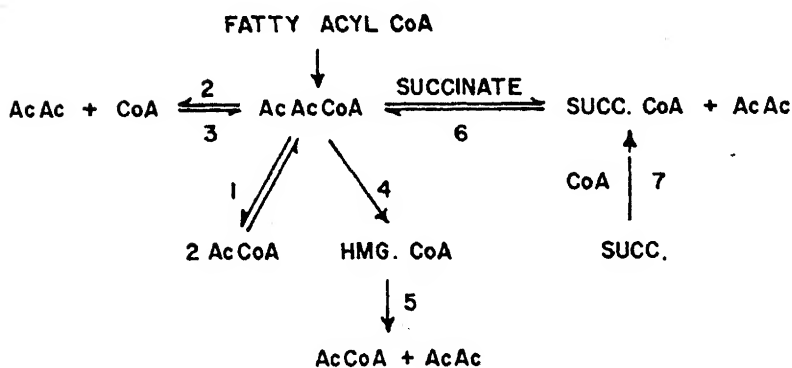
Substrate	CPM incorporated		PA	PC
	Total lipids	Neutral lipids		
Palmitoyl + oleyl CoA	984 ± 25 <sup>a</sup>	426 ± 20	144 ± 7	293 ± 6 <sup>c</sup>
Palmitoyl + oleyl DTO	1332 ± 77 <sup>b</sup>	444 ± 34	176 ± 31	480 ± 26 <sup>d</sup>

All values are means of 5 independent determinations.

<sup>b</sup>Significantly higher than <sup>a</sup>  $P < 0.01$ .

<sup>d</sup>Significantly higher than <sup>c</sup>  $P < 0.001$ .

Rat liver microsomes were isolated according to Yamashita and Numa (1981). Incorporation of  $[U-^{14}C]$ -Sn-glycerol-3-phosphate into triglyceride and phospholipids in rat liver microsomes was carried out according to Schneider (1963). The assay mixture in a final volume of 0.4 ml contained 1.2 m mol of Tris-HCl buffer pH 8.0, 0.1  $\mu$ mol phosphoryl choline 0.2  $\mu$ mol CTP, 0.3  $\mu$ mol ATP, 6 mg glycerol 1-2  $\mu$ mol  $\beta$ -mercaptoethanol, phosphoryl choline cytidyl transferase (1 mg protein). The mixture was incubated for 30 min and 30 n mol of palmitoyl CoA and oleyl CoA or 30 n mol of palmitoyl DTO and 31 n mol of oleyl DTO was added. The mixture was incubated for a further 30 min, extracted with chloroform methanol 2:1 v/v and the fractions of neutral and phospholipids separated by silicic acid column chromatography (Sweeley, 1969). The fractions were counted for radioactivity.



1.  $\beta$ -KETO THIOLASE

2. ACETOACETYL CoA HYDROLASE

3. ACETOACETATE ACTIVATING ENZYME

4. HMG CoA SYNTHASE

5. HMG CoA LYASE

6. SUCCINYL CoA : 3 OXO ACID CoA  
TRANSFERASE

7. SUCCINYL CoA SYNTHETASE

molecules of acetyl CoA and from the acetyl

Table 4 shows that DTO does not participate in the thiolase reaction, for example, there is no acetyl DTO formation from acetoacetyl DTO + DTO. On the other hand, DTO seems to participate albeit to a very small extent in the mixed thiolase reaction like acetoacetyl CoA + DTO or acetoacetyl DTO + CoA. These findings support the effect of DTO in reducing blood acetoacetate levels and point to its usefulness in the prevention of acidosis in diabetes.

**Table 4.** Thiolysis of acetoacetyl DTO.  
 $\text{AcAcCoA} + \text{CoA} \rightleftharpoons 2 \text{ AcCoA}$

Substrate	Thiol	Sp. activity (Units/mg protein)
AcAcCoA	CoA	5.0
	DTO	0.6
AcAcDTO	CoA	0.2
	DTO	ND*

Thiolase was partially purified from normal rat liver mitochondria. The enzyme was assayed by monitoring the decrease in  $A_{310}$  absorption which is an indication of the disappearance of acetoacetyl thioester. One unit of activity corresponds to a decrease in  $A_{310}$  of 0.01/min.

\*Not detectable.

**Table 5.** Succinyl CoA:3 oxo acid transferase.  
 $\text{Succinyl CoA} + \text{AcAc} \rightleftharpoons \text{AcAcCoA} + \text{Succinate}$ .

Substrate	Activity	
	$A_{310}/\text{min/mg}$ protein	$\mu\text{mol of substrate converted/min/mg}$ protein
AcAc CoA	0.7	0.18
AcAc DTO	ND	ND

ND, Not detectable.

The enzyme was isolated from rat hearts according to Stern *et al.* (1956). The enzyme was assayed by monitoring the disappearance of acetoacetyl thioester, on addition of succinate, by the decrease in  $A_{310}$  absorbance.

Further, as shown in table 5, acetoacetyl DTO does not lead to the formation of acetoacetate by the thiophorase reaction using succinate. Acetoacetyl DTO therefore does not contribute to ketone body formation *via* thiophorase reaction. Further, acetoacetyl DTO may have a feedback inhibition effect on fatty acid oxidation thus ensuring reduced catabolism of fat especially in conditions like diabetes.

HMG.DTO is formed from acetoacetyl DTO and acetyl DTO to about half the level of HMG.CoA from acetoacetyl CoA and acetyl CoA (table 6).

Another metabolic reaction of importance is the formation of dicarboxylate thioesters. Succinyl CoA synthetase (EC 6.2.1.4) was isolated from rat hearts according to Murakami and Nishimura (1974). The assay was carried out by incubating succinate and CoA or DTO and following the increase in absorbance at 235 nm

Table 6. Formation of HMG:CoA/DTO catalyzed by HMG:CoA synthetase

Source	Substrate	n mol of HMG:CoA produced/ min/mg protein	
Cytosol	AcAcCoA	AcCoA	3.2
		AcDTO	ND
		AcCoA	ND
	AcAcDTO	AcDTO	1.2
		AcCoA	3.4
Mitochondria	AcAcCoA	AcDTO	ND
		AcCoA	ND
		AcDTO	0.83
	AcAcDTO		

ND, Not detectable.

The enzymes were isolated from rat liver cytosol or mitochondrial fractions according to Clinkenbeard *et al.* (1980). The assay mixture in a final volume of 1 ml contained 100  $\mu$ mol Tris HCl, 0.1  $\mu$ mol EDTA, 0.2  $\mu$ mol acetyl CoA or acetyl DTO, 0.05  $\mu$ mol acetoacetyl CoA or acetoacetyl DTO, 20  $\mu$ mol MgCl<sub>2</sub> and 200  $\mu$ g enzyme protein. The disappearance of acetoacetyl CoA or acetoacetyl DTO was monitored spectrophotometrically at 310 nm.

CoA. Succinyl CoA is also formed by the decarboxylation of  $\alpha$ -keto glutarate even as acetyl CoA is formed by the oxidation of pyruvate.

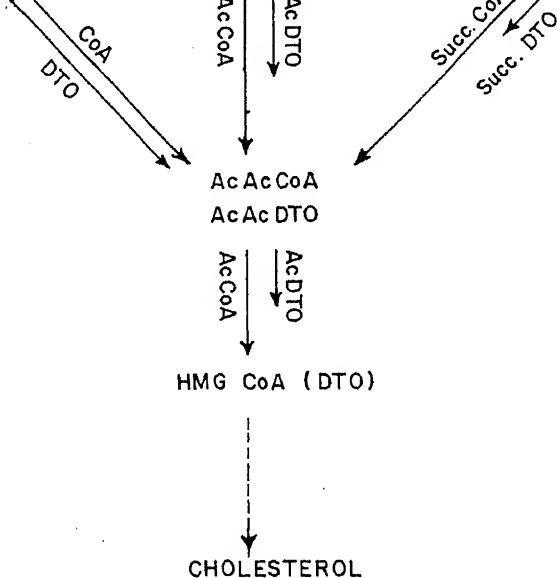
It may be worthwhile pointing out that in the thiolase reaction acetoacetyl DTO was ineffective. Further, whereas in mixed thiolase reaction DTO evinced low activity levels, it had no effect at all in HMG formation. The summary of our investigation on acetoacetate metabolism is presented in figure 3. The length of the arrows represent the ratio of the activity of CoA and DTO derivatives. It will be seen from figure 3 that DTO reduces the formation of acetoacetyl DTO and HMG.DTO precursors for biosynthesis of cholesterol because of reduced thiolase activity and reduced thiophorase activity. On the other hand, the removal of ketosis, that is, conversion of acetoacetate to acetoacetyl DTO is well performed by DTO. So taking the 4 major reactions leading to ketogenesis and cholesterologenesis it may be stated on balance that DTO would help reduction of both.

#### Effect of lipoic acid on [U-<sup>14</sup>C]-glucose and [1-<sup>14</sup>C]-palmitic acid oxidation in intact rats

The reactions of DTO and acetyl DTO in various enzyme systems subtly alters the overall utilization of carbohydrates and fat in diabetic rats. In order to establish this, the oxidation of [U-<sup>14</sup>C]-glucose and [1-<sup>14</sup>C]-palmitic acid in normal and diabetic rats following lipoic acid administration was measured. These results were compared with those obtained by the administration of insulin and glibenclamide (an oral hypoglycaemic drug).

Tables 7 and 8 show the effect of intraperitoneal administration of lipoic acid and





Length of arrows indicate approx. relative rates

Figure 3. Acetoacetate metabolism.

Table 7.  $^{14}\text{CO}_2$  expiration from  $[\text{U-}^{14}\text{C}]$ -glucose.

Animals	CPM $^{14}\text{CO}_2$ /ml $\text{CO}_2$ /sq. m. area
Normal (6)	$97 \pm 10^a$
Normal + Lipoic acid (6)	$96 \pm 9^b$
Diabetic (12)	$51 \pm 4^c$
Diabetic + Lipoic acid (5)	$77 \pm 11^d$
Diabetic + Insulin (6)	$91 \pm 5^e$
Diabetic + Glibenclamide (7)	$69 \pm 3^f$

Numbers in parentheses indicate the number of animals. Lipoic acid (100 mg/kg) and insulin (40 i.u./kg) were administered intraperitoneally. Glibenclamide (5 mg/kg) was administered orally.  $[\text{U-}^{14}\text{C}]$ -glucose ( $1.5 \times 10^5$  cpm) was injected intraperitoneally 2 h later and the expired  $\text{CO}_2$  was trapped in KOH containing vials and counted.

<sup>a</sup> Not significantly different from <sup>a</sup>.

<sup>c</sup> Significantly less than <sup>a</sup>  $P < 0.001$ .

<sup>d</sup> Significantly greater than <sup>c</sup>  $P < 0.01$ .

<sup>e</sup> Significantly greater than <sup>c</sup>  $P < 0.001$ .

<sup>f</sup> Significantly greater than <sup>c</sup>  $P < 0.01$ .

glucose and  $[\text{1-}^{14}\text{C}]$ -palmitic acid. Oxidation of glucose which is impaired in diabetes is increased by insulin and lipoic acid and to a smaller extent by glibenclamide. Oxidation of  $[\text{1-}^{14}\text{C}]$ -palmitic acid which is increased in diabetes is reduced by insulin and lipoic acid and not by glibenclamide. Thus, the often small changes

Animals	CPM $^{14}\text{C}$ CO <sub>2</sub> /ml CO <sub>2</sub> /sq. m. surface area
Normal (5)	22 ± 1 <sup>a</sup>
Diabetic (12)	47 ± 2 <sup>b</sup>
Diabetic + Lipoic acid (4)	36 ± 3 <sup>c</sup>
Diabetic + Insulin (5)	42 ± 2 <sup>c</sup>
Diabetic + Glibenclamide (6)	46 ± 2 <sup>c</sup>

Numbers in parentheses denote the number of animals. Lipoic acid (100 mg/kg) and insulin (40 i.u/kg) were administered intraperitoneally. Glibenclamide (5 mg/kg) was administered orally.  $1\text{-}^{14}\text{C}$  palmitic acid ( $1.5 \times 10^5$  cpm) was injected intraperitoneally two hours later and the expired CO<sub>2</sub> was trapped in vials containing KOH and counted.

<sup>b</sup> Significantly greater than <sup>a</sup>  $P < 0.01$ .

<sup>c</sup> Significantly less than <sup>b</sup>  $P < 0.01$ .

<sup>d</sup> and <sup>c</sup> not significantly different from <sup>b</sup>.

obtained by substituting CoA with DTO add up to produce a substantial change in the overall homeostasis in diabetes. We believe that these effects are responsible for the beneficial effects of lipoic acid administration in diabetes.

## Metabolism of acetyl DTO

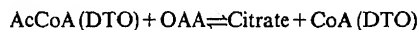
While DTO can substitute for CoA in a wide array of enzymes involved in fatty acid oxidation and biosynthesis of fatty acids, triglycerides and phospholipids, it is interesting to note that acetyl DTO can also substitute for acetyl CoA in a number of enzymic reactions such as biosynthesis of malonyl DTO and HMG.DTO (vide supra), citrate synthesis, and participation in transacetylation reactions with thioesters of CoA (Brady and Stadman, 1954).

As can be seen from table 9, acetyl DTO has about 40% of the specific activity of acetyl CoA for citrate synthase (Gandhi *et al.*, 1985).

It is not clear whether sufficient quantities of acetyl DTO are formed physiologically to warrant considerations of its oxidation through citric acid cycle. Nevertheless, it is important to note in this context that lipoic acid administration increases glucose oxidation.

Table 9. Metabolism of acetyl DTO.

Citrate synthase



Substrate	OD/min at 412 nm	Sp. activity*
AcCoA	0.074	0.82
AcDTO	0.027	0.30

\* $\mu$  mol of thiol released/min/mg protein.

The enzyme was isolated from normal rat liver mitochondria and partially purified. The enzyme was assayed by estimating the free thiol formed using

DTO by a lipoyl transacetylase (EC 2.3.1.12) reaction can convert acetyl CoA to acetyl DTO. This enzyme is present in normal rat liver and has been partially purified. It was found that the enzyme had a specific activity of 0.4 n mol acetyl DTO formed/min/mg protein (Gandhi *et al.*, 1985).

### Biosynthesis of lipoic acid

The precursor for the biosynthesis of lipoic acid has been shown to be octanoic acid in *Escherichia coli* (White, 1980 a,b, 1981). Carreau *et al.* (1977) have shown that linoleic acid and to a smaller extent oleic acid act as precursors for lipoic acid biosynthesis in the rat. The reaction has been shown to occur predominantly in the microsomal fraction of rat liver (Spoto *et al.*, 1982). We have found (table 11) that arachidonic acid appears to be the immediate precursor followed by linoleic acid for the biosynthesis of lipoic acid in normal and diabetic rats. Furthermore, in diabetic liver, biosynthesis of lipoic acid from linoleic acid is impaired and addition of insulin to the perfusion medium corrects this (table 12). It appears therefore, that in alloxan

**Table 10.** Radioimmunoassay of serum insulin in normal and diabetic rats.

Rats	Insulin levels ( $\mu$ IU/ml of serum)	Blood Glucose (mg/100 ml)
Normal (20)	59.6 $\pm$ 7.9 <sup>a</sup>	110 $\pm$ 5 <sup>b</sup>
Normal + Lipoic acid (20)	52.8 $\pm$ 8.2	90 $\pm$ 5 <sup>c</sup>
Diabetic (12)	31.6 $\pm$ 4.2 <sup>d</sup>	338 $\pm$ 55
Diabetic + Lipoic acid (12)	29.3 $\pm$ 3.5	288 $\pm$ 52

<sup>a</sup> Significantly less than <sup>a</sup>  $P < 0.05$ .

<sup>c</sup> Significantly less than <sup>b</sup>  $P < 0.02$ .

Rats were sacrificed 1 h after intraperitoneal injection of lipoic acid at 10 mg/100 g. Insulin levels were estimated using radioimmunoassay kit supplied by Bhabha Atomic Research Centre, Bombay, following the procedure outlined therein.

**Table 11.** Incorporation of 1-[<sup>14</sup>C]-labelled compounds into liver fat and lipoic acid.

Labelled precursor	Rats	Amount perfused in liver cpm ( $\times 10^6$ )	Radioactivity incorporated in fat		Radioactivity recovered in TLC fraction		Radioactivity recovered in HPLC-lipoic acid	
			cpm ( $\times 10^6$ )	per cent	cpm ( $\times 10^3$ )	per cent	cpm ( $10^3$ )	per cent
Arachidonic acid	N	1.9	1.06	55.6	16.6	0.87	2400	0.125
	D	1.7	0.690	40.3	6.84	0.398	560	0.032
Octanoic acid	N	3.2	NE	NE	23.56	0.73	3520	0.109
	D	4.3	0.34	7.9	8.05	0.185	508	0.012
Linoleic acid	N	8.18	2.59	31.7	48.20	0.59	6650	0.081
	D	4.8	0.89	18.4	0.59	0.012	ND	ND
Palmitic acid	N	8.0	0.15	1.94	0.10	0.001	ND	ND

Table 12. Incorporation of 1-[ <sup>14</sup>C]-linoleic acid into lipoic acid in presence of insulin.

Rats	Amount perfused (× 10 <sup>6</sup> )	Radioactivity (cpm)			Per cent incorporation	
		Fat (× 10 <sup>6</sup> )	Lipoic (TLC)	Lipoic (HPLC)	Fat	Lipoic (HPLC)
Normal	5.27	1.72	27,000	3,800	32.6	0.072
Diabetic	3.63	0.54	6,100	ND	14.4	ND
Diabetic + insulin	2.24	0.8	3,500	810	35.7	0.036

ND, Not detectable.

Livers were perfused with the labelled precursor for 4 h and incubated for 3 h at room temperature. In experiments with insulin, 0.25 i.u/ml of insulin was used in the perfusion medium. Lipoic acid was isolated and purified as described in table 11.

diabetes, the biosynthesis of lipoic acid may be impaired, primarily due to the reduction in the conversion of linoleic to arachidonic acid (Brenner and Peluffo, 1969).

### Does lipoic acid stimulate insulin secretion?

Previous studies (Natraj *et al.*, 1984) have shown that lipoic acid administration at 10 mg/100 g in alloxan diabetic rats resulted in beneficitation of the following biochemical lesions:

- Serum pyruvate normalised in 1 h.
- Liver pyruvate dehydrogenase normalised in 1 h.
- Blood sugar reduced by 40% in 4 h.
- Serum acetoacetate decreased by a 3 fold margin in 24 h.
- Liver glycogen increased by a 2 fold margin in 24 h.
- Liver fatty acid synthesis increased by a 2 fold margin in 24 h.

These are similar to the effect of insulin administration. Lipoic acid on reduction forms DTO which has two sulphhydryl groups and sulphhydryl containing compounds *in vitro* have been shown to stimulate insulin secretion (Haugaard and Haugaard, 1970; Lavis and Williams, 1970). Hence there is always the question whether the beneficial effects observed due to administration of lipoic acid are really due to lipoic acid *per se* or intermediated through insulin response. Table 10 shows that insulin levels in diabetes or in the normal state is uninfluenced by the administration of lipoic acid indicating that the effect of lipoic acid is specific and not mediated through insulin.

### An overview of research on lipoic acid and diabetes

In figure 4, we have given a list of reactions where DTO substitutes for CoA completely or partially. These are acetate, acetoacetate, succinate, malonate and fatty

# DTO REPLACES CoA IN THE FOLLOWING REACTIONS

- FATTY ACID + DTO + ATP  $\longrightarrow$  FATTY ACYL DTO
- ACETATE + DTO + ATP  $\longrightarrow$  AcDTO
- AcAc + DTO + ATP  $\longrightarrow$  Ac Ac DTO
- SUCC. + DTO + ATP  $\longrightarrow$  SUCC. DTO
- MALONATE + DTO + ATP  $\longrightarrow$  MAL DTO
- PYR. + DTO +  $\text{NAD}^+$  +  $\text{H}^+$   $\longrightarrow$  AcDTO + NADH
- AcDTO + OXALACETATE  $\longrightarrow$  CITRATE + DTO
- AcDTO +  $\text{HCO}_3^-$  + ATP  $\longrightarrow$  MAL DTO
- AcDTO + AcAcDTO  $\longrightarrow$  HMG DTO
- L-GLY.-3-P + FATTY ACYL DTO  $\longrightarrow$  TG + PL

Figure 4. Comparison of DTO and CoA.

biosynthesis and pyruvate oxidation. In many of the reactions where DTO replaces CoA the rates of the reaction are of the order of 50% of CoA.

Figure 5 indicates reactions where DTO does not substitute for CoA. These are thiolase, thiophorase and pyruvate carboxylase reactions. The non-participation of DTO in these 3 reactions is purposeful in reduction of ketogenesis and gluconeogenesis.

## DTO DOES NOT REPLACE CoA IN THE FOLLOWING REACTIONS :

- AcAcDTO  $\nrightarrow$  2 AcDTO
- PYR +  $\text{HCO}_3^-$  + AcDTO + ATP  $\nrightarrow$  OXALACETATE
- AcAc + SUCC. DTO  $\nrightarrow$  AcAcDTO + SUCC.

Figure 5. Comparison of CoA and DTO.

As can be seen from the results described so far, most enzymatic reactions involving DTO or acetyl DTO are less active as compared to CoA or acetyl CoA. One reason for this could be that the DTO used was a racemic mixture and the acetyl<sub>4</sub>DTO is a mixture of 4 possible isomers (+) and (-) 6-S-acetyl and (+) and (-) 8-S-acetyl derivatives. If only one of these derivatives is the actual substrate for the enzymatic reactions, then the actual concentration of active acetyl DTO would be a fourth of the applied concentration. This indeed, was found to be the case for citrate synthase wherein, the  $K_m$  for acetyl DTO was 4 times higher than for acetyl CoA (Wagh *et al.*, 1986). It has been reported that acylation of the lipoic acid residues in the enzyme complex of pyruvate dehydrogenase is asymmetric with the 8-S-acetyl derivative being the first product (O'Connor *et al.* 1982).

pathways for the biosynthesis of fatty acids, triglycerides and phospholipids. Although, the effects of lipoic acid have been demonstrated in diabetic animals as a model system, the significance of these findings transcends the confines of this disease state and are relevant to the normal animal as well. The results obtained with triglyceride and phospholipid biosynthesis where fatty acyl DTO is in fact a better substrate than fatty acyl CoA merits special attention, since it raises the question as to whether in the normal animal activation of fatty acids for triglyceride and phospholipid biosynthesis occurs with the help of thiols other than CoA. It may be recalled that in the oxidation of fatty acids the requirement for CoA as the thiol component appears to be absolute. It is in keeping with nature's way of controlling and differentiating metabolic fluxes, to propose that CoA thioesters of fatty acids are primarily utilized for oxidation and DTO thiesters for lipid biosynthesis.

The role of essential fatty acids in various biochemical processes such as precursors for thromboxanes, prostaglandins etc. and as integral components of phospholipids in the membranes where, by virtue of their lower melting temperatures they confer a higher degree of fluidity to the membrane has been well established. It has also been shown (Houtsmuller *et al.*, 1981) that essential fatty acids have an insulin sparing action in diabetes. The mechanism of this however is not clear. It is possible that increased fluidity of membranes may influence membrane processes such as transport, hormone receptor interaction, signal transduction etc. which individually or collectively may be responsible for the observed effects of essential fatty acids in diabetes. An even more intriguing angle to this is the conversion of essential fatty acids and particularly arachidonic acid to lipoic acid (Menon and Natraj, 1984). The fact that arachidonic acid levels are reduced in diabetes and that linoleic acid to arachidonic acid conversion is insulin dependent raises the question whether a part of the problem in diabetes is due to the reduced formation of lipoic acid.

In summary, the role of lipoic acid in intermediary metabolism may not be confined to its cofactor role in  $\alpha$ -keto acid dehydrogenases but may well extend to certain reactions especially in lipid biosynthesis where, it may replace CoA for activating fatty acids prior to acylation with L-glycerol-3 phosphate. The significance of these reactions will become clearer if lipoic acid can sustain growth of pantho-theine requiring mutants in the absence of panthoetheine.

## References

- Brady, R. O. and Stadman, E. R. (1954) *J. Biol. Chem.*, **211**, 621.  
 Brenner, R. R. and Peluffo, R. O. (1969) *Biochim. Biophys. Acta*, **170**, 471.  
 Carreau, J. P., Lapous, D. and Raulin, J. (1977) *Biochimie*, **59**, 487.  
 Cate, R. L. and Roche, T. E. (1983) *J. Biol. Chem.*, **258**, 4771.  
 Chiles, R. and Tzagournis (1970) *Diabetes*, **19**, 458.  
 Clinkenbeard, K. D., Sugiyama, T. and Lave, D. M. (1980) *Methods Enzymol.*, **35**, 160.  
 Foster, D. W. (1983) in *The metabolic basis of inherited disease* (eds J. B. Stansbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein and M. S. Brown) (New York: McGraw Hill) p. 99.  
 Gandhi, V. M., Wagh, S. S., Natraj, C. V. and Menon, K. K. G. (1985) *J. Biosci.*, **9**, 117.  
 Gerard, R., Guy, V., Loubatieres, M. and Madeline, M. (1979) *Diabetes*, **28**, 852.  
 Haugaard, N. and Haugaard, E. S. (1970) *Biochim. Biophys. Acta*, **222**, 583.  
 Hersh, L. B. and Jencks, W. P. (1967) *J. Biol. Chem.*, **242**, 3468.

- Jones, M. E. and Lipmann, F. (1955) *Methods Enzymol.*, **1**, 585.
- Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, A. T. and Denton, R. M. (1976) *Biochem. J.*, **154**, 327.
- Lavis, V. R. and Williams, R. H. (1970) *J. Biol. Chem.*, **245**, 23.
- Menon, K. K. G. and Natraj, C. V. (1984) *J. Biosci.*, **6**, 459.
- Moore, S. A. and Jencks, W. P. (1982) *J. Biol. Chem.*, **257**, 10893.
- Murakami, Y. and Nishimura, J. S. (1974) *Biochim. Biophys. Acta*, **336**, 252.
- Natraj, C. V., Gandhi, V. M. and Menon, K. K. G. (1984) *J. Biosci.*, **6**, 37.
- Neufeld, M., Maclaren, N. K., Riley, W. J., Lezotte, D., McLaughlin, J. Y., Silverstein, J. and Rosenbloom, A. L. (1980) *Diabetes*, **29**, 589.
- O'Connor, T. P., Roche, J. E. and Paukstelis, J. V. (1982) *J. Biol. Chem.*, **257**, 3110.
- Ohgaku, S., Brady, P. S., Schuman, W., Bartch, G. E., Margolis, J. M., Kumaran, K., Landau, S. B. and Landau, B. R. (1983) **258**, 9283.
- Olefsky, J. M. and Kotterman, O. G. (1981) *Am. J. Med.*, **70**, 151.
- Sauer, F. and Erfle, J. D. (1966) *J. Biol. Chem.*, **241**, 30.
- Schneider, N. C. (1963) *J. Biol. Chem.*, **238**, 3572.
- Segal, H. L. and Menon, K. K. G. (1961) *J. Biol. Chem.*, **236**, 2872.
- Spoto, G., Dupre, S. and Cavallini, D. (1982) *IRCS Med. Sci.*, **10**, 626.
- Stackpole, P. W., Moore, G. W. and Kornhauser, D. M. (1978) *New Engl. J. Med.*, **298**, 526.
- Stern, J. R., Coon, M. J., Delcampillo, A. and Schneider, N. C. (1956) *J. Biol. Chem.*, **231**, 15.
- Stoops, J. K., Ross, P., Arslanian, M. J., Aune, K. C. and Wakil, S. J. (1979) *J. Biol. Chem.*, **254**, 7418.
- Sweeley, C. (1969) *Methods Enzymol.*, **14**, 45.
- Utter, M. F., Keech, D. B. and Scrutton, M. C. (1964) *Adv. Enzyme Regul.*, **2**, 49.
- Wagh, S. S., Gandhi, V. M., Natraj, C. V. and Menon, K. K. G. (1986) *J. Biosci.*, **10**, 171.
- Weinberg, M. B. and Utter, M. F. (1980) *Biochem. J.*, **188**, 601.
- Wieland, O., Siess, E., Shulze-Wethmar, F. A., von Funcke, H. G. and Winton, B. (1971) *Arch. Biochem. Biophys.*, **143**, 593.
- White, R. H. (1980a) *Biochemistry*, **19**, 15.
- White, R. H. (1980b) *J. Am. Chem. Soc.*, **102**, 6605.
- White, R. H. (1981) *Anal. Biochem.*, **110**, 89.
- Whitehouse, S. and Randle, P. J. (1973) *Biochem. J.*, **134**, 651.
- Yamashita, S. and Numa, S. (1981) *Methods Enzymol.*, **71**, 552.
- Zadik, Z., Kayne, R., Kappy, M., Plotnick, L. P. and Kowarski, A. A. (1980) *Diabetes*, **29**, 655.

# How does cobalamin (vitamin B12) enter and traverse mammalian cells?

BELLUR SEETHARAM

Department of Medicine, Division of Gastroenterology, Washington University School of Medicine, St. Louis, Missouri 63110, USA

**Abstract.** This overview is intended to present recent information available on the work done in understanding the mode of entry of cobalamin into and out of mammalian cells. I have focused primarily on the uptake of cobalamin bound to 3 proteins: intrinsic factor, transcobalamin II and R-proteins. Even though the nature of these cobalamin binding proteins, and the receptors involved in the internalization of these proteins is known, a total picture on the intracellular trafficking of cobalamin and the binding proteins is far from complete.

**Keywords.** Cobalamin; uptake; cells; receptor; endocytosis; acidic vesicles.

## Introduction

Cobalamin (cbl) a water soluble micronutrient, is taken up by mammalian cells across the lipid plasma membranes by receptor mediated endocytosis, a process which is common for the cellular utilization of proteins and peptides. Rapid strides have been made in our current understanding of how the various ligands such as peptide hormones, low density lipoproteins and others are taken up and sorted in the cells. However, such a detailed information on the uptake and cellular sorting of cbl is lacking mainly due to the complex nature of cbl and the proteins that mediate uptake. Despite this, work done in our laboratory and others have provided important clues regarding these processes.

## Early events before absorption

In mammalian systems the only source of this vitamin is through diet, and the first cells which has to take up this vitamin are the ileal cells of intestinal mucosa. The sequence of events which lead up to the entry of cbl into blood is truly a complex, yet remarkably efficient process (Seetharam *et al.*, 1982, 1985). Our current knowledge has come from *in vivo* and *in vitro* studies using various animal model systems. A very tiny amount (2–3  $\mu\text{g}$ ) of the vitamin is absorbed in humans at any given time. Before this amount can be absorbed by the ileal cells a series of complex binding reactions occur in the stomach and the intestinal lumen. The dietary cbl which is mostly bound to proteins in the food is released by the action of acid and pepsin. Following this release cbl binds very strongly to a protein which is released from the salivary glands. Once cbl is bound to this protein, it can be released only when the protein is degraded by proteases. The cbl bound to the salivary binder cannot be utilized by the ileal cells. The reason for this paradox is due to the fact that in the

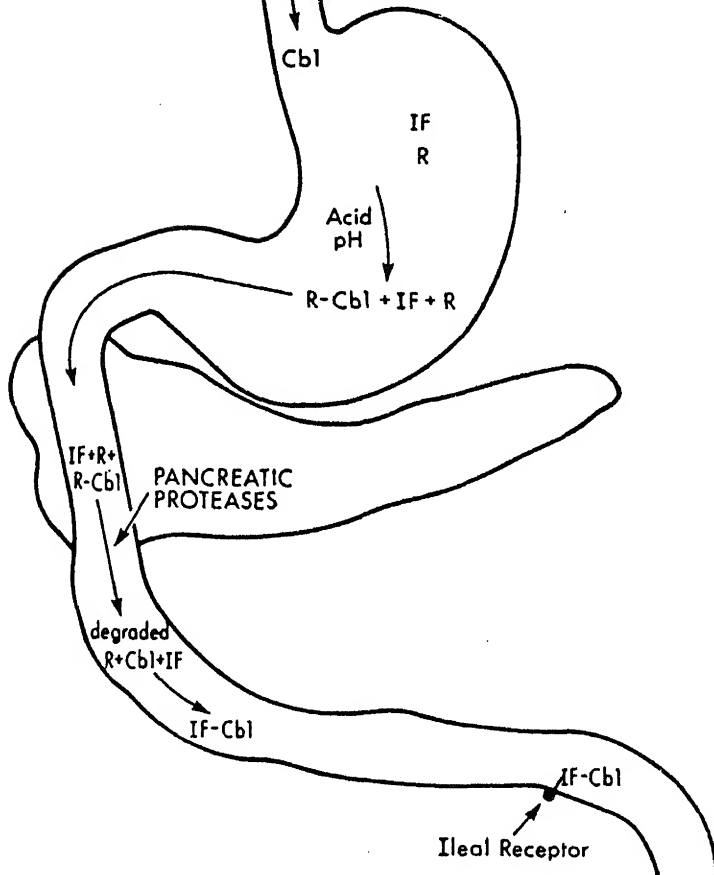


acid milieu of the stomach salivary binder has an affinity for cbl which is 50 times higher than for intrinsic factor, a glycoprotein secreted from the parietal cells of the stomach (Allen and Mehlmán, 1973). Cbl bound to gastric intrinsic factor is very efficiently taken up by the ileal cells. During the normal course of events the dietary cbl which binds to salivary binder must be transferred to gastric intrinsic factor before it can be absorbed. Failure in such a transfer results in cbl malabsorption. Allen *et al.* (1978a) showed that cbl bound to salivary binder can be transferred to gastric intrinsic factor in the presence of pancreatic proteases such as trypsin, chymotrypsin and elastase. This simple *in vitro* demonstration helped provide a biochemical explanation for the cause of malabsorption of cbl in patients with pancreatic insufficiency (Toskes *et al.*, 1971). Allen *et al.* (1978b) provided further *in vivo* evidence, for their *in vitro* observations when they corrected malabsorption of cbl in patients with pancreatic insufficiency either by oral administration of trypsin or an analog of cbl which bound to only salivary binder but not to gastric intrinsic factor. A schematic diagram of the sequence of events mentioned above is shown in figure 1.

### Intestinal uptake of IF-cbl

Once cbl complexes with intrinsic factor, the complex attaches to a receptor which for some strange reason is located in the distal ileum in most animal species. This binding requires  $\text{Ca}^{2+}$  and a pH above 5 and below 7.5. The receptor which recognizes the ligand (IF-cbl) has been purified to homogeneity (Seetharam *et al.*, 1981), from canine ileal mucosa. The receptors purified from hog and human (Kouvonen *et al.*, 1979) and canine ileal mucosa (Seetharam *et al.*, 1981) have a molecular weight ( $M_r$ ) around  $230\text{--}240 \times 10^3$  and consist of two subunits. The canine receptor is located in the microvillus pits (Levine *et al.*, 1984) and the IF-cbl binding domain is located on the luminal side of the membrane (Seetharam *et al.*, 1981b). The canine receptor is anchored to the plasma membrane *via* an anchor piece which is rather large ( $M_r$  36,000) (Seetharam *et al.*, 1982). Not all mammalian IF-cbl receptors are the same. The rat receptor does not cross-react with antiserum to canine receptor and can bind cbl only when complexed with rat IF but not IF prepared using human, hog and canine stomachs (Seetharam *et al.*, 1983).

Following the attachment of IF-cbl complex to the ileal receptor how the vitamin gets in and gets out of the ileal cell is not fully understood. However, it is fairly clear that IF-cbl complex is internalized (Kapadia *et al.*, 1983). Following this internalization analysis of intracellular cobalamin has revealed that cbl is bound to free IF, If which is still attached to the receptor, and finally to a transport protein (Transcobalamin II; TC-II) which shuttles cbl to various tissues. The exact vesicle where the release of cbl occurs, and the mechanisms by which this release is brought about has been a subject of intensive research. Most ligands which enter a cell by receptor mediated endocytosis are initially present in the endocytic vesicles. These vesicles are acidic and are called receptosomes (Willingham and Pastan, 1980). Most ligands are dissociated from their receptor due to low pH of these prelysosomal vesicles. The receptor in some cases recycles to the membrane or it can get degraded. The ligands are delivered *via* golgi to the lysosomes for eventual degradation. In some cases both

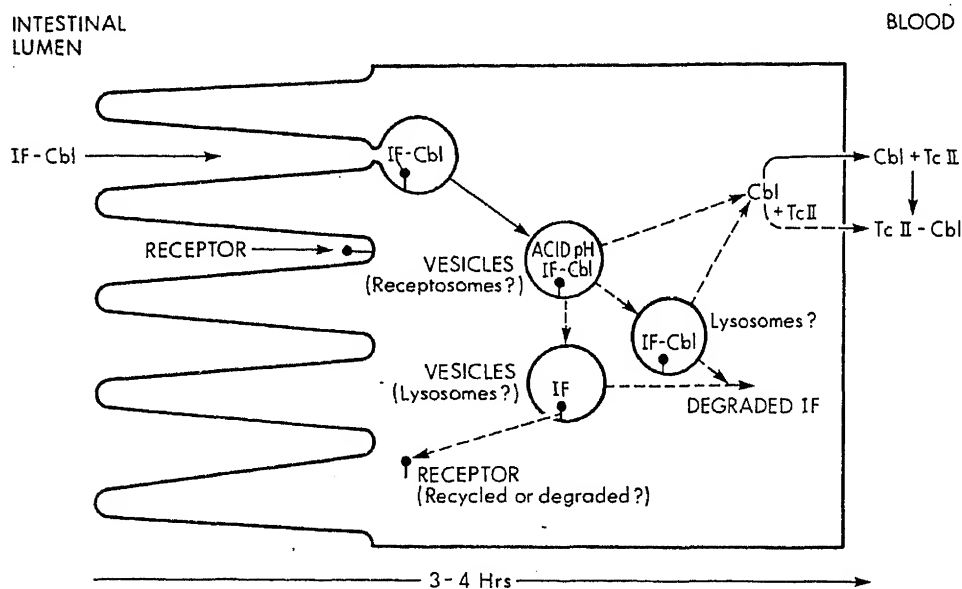


**Figure 1.** Proposed scheme for the absorption of dietary cobalamin. Reproduced with permission from Annual Reviews of Nutrition: Seetharam and Alpers (1982a).

Once IF-cbl is internalized the intracellular sorting which results in the transfer of cbl from IF to TC-II is a complex process and is poorly understood. Our areas of ignorance include the following: (a) the intracellular vesicle (endosomes or lysosomes) where the actual release of cbl occurs; (b) the mechanism (proteolytic cleavage or acidic pH) by which this release occurs; (c) the fate of IF and its receptor (degraded or recycled) and finally the origin of TC-II (mucosal or blood).

The answers to some of these questions have begun to emerge. Recent work by Seetharam *et al.* (1985) and Robertson *et al.* (1985a,b) have suggested that cbl is probably released due to acidification of IF-cbl complex and not due to proteolytic degradation of IF. In the presence of chloroquine there was an inhibition in the amount of cbl transferred to TC-II from IF suggesting that acid alone can bring about this transfer. Even though cbl bound to TC-II has been demonstrated in the

mucosal extracts, following gastrointestinal uptake of cbl, a direct demonstration of a *de novo* synthesis of TC-II or its presence in a cell free homogenate in the absence of IF-cbl uptake has never been demonstrated. In direct evidence (Chanarin *et al.*, 1978; Rothenberg *et al.*, 1978) has suggested that TC-II may be produced by ileal cells in response to the uptake of IF-cbl by these cells. A schematic diagram showing the uptake of IF-cbl by the enterocytes and the areas of ignorance is shown in figure 2.



**Figure 2.** Possible pathways for movement of IF and cbl within the enterocyte. Established pathways are marked by solid lines. Pathways that are incompletely established or proposed are marked by broken lines.

### Cellular uptake of TC-II-cbl

The cbl which exists the ileal mucosa must be delivered to the tissues. This is accomplished by TC-II, a protein of  $M_r$  38,000. Cobalamin bound to TC-II is taken up by receptor mediated endocytosis *via* a receptor which is located in the plasma membrane of all tissues. The receptor for TC-II has been purified from human placenta (Seligman and Allen, 1978). The receptor has a  $M_r$  50,000 and has very similar amino acid composition as its ligand TC-II, suggesting the possibility that TC-II and its receptor might have originated from a common gene. The TC-II receptor binds both apo TC-II and holo TC-II, but the association constant ( $k_a$ ) for holo TC-II is twice that of apo TC-II. The plasma clearance (Schnider *et al.*, 1976) of [ $^{125}$ I]-TC-II was 30% faster than the clearance for [ $^{125}$ I]-TC-II-cbl; suggesting that *in vivo* apo TC-II may have higher affinity for the receptor than holo TC-II. This discrepancy might simply reflect the results one might obtain when one calculates

fibroblasts (Turner *et al.*, 1978), and rat kidney (Newmark *et al.*, 1970).

### **Hepatic uptake of cbl bound to nonintrinsic factor (R-proteins)**

In addition to IF and TC-II a family of cobalamin binding proteins have been identified and in some cases been purified. These proteins have been identified in leukocytes, plasma and other biological fluids. These proteins are all immunologically identical, but because of their rather rapid mobility during electrophoresis, they are called R-proteins. The R-proteins purified from human milk, saliva and normal granulocytes have a  $M_r$  around  $60-66 \times 10^3$ .

The physiological significance for the presence of R-proteins in mammalian secretion is not clear. It has been suggested (Allen, 1975) that potentially harmful analogs of cbl (which might be present either in the diet or produced by bacteria), are cleared by binding with very high affinity to these proteins. The cbl or cbl analogs bound to R-proteins is cleared in the liver *via* asialoglycoprotein receptor which is present in the hepatic plasma membrane. This receptor recognizes glycoprotein ligands once their sialic acid is removed, exposing the terminal galactose (Ashwell and Morell, 1974). The plasma clearance of cbl bound to R-proteins is very fast ( $T_{1/2} = 5$  min) (Burger *et al.*, 1975). The cbl or their analogs are released in the lysosomes and secreted through the bile. When the cbl analogs are cleared through bile, they are not reabsorbed since intrinsic factor binds these analogs with lower affinity than cbl.

### **Cobalamin deficiency due to defective intracellular sorting of cbl**

Cbl deficiency in man and other animals can occur due to a number of causes (Kapadia *et al.*, 1985) during its complicated pathway of absorption, transport and cellular utilization (Rosenberg 1983). In addition to these causes cbl deficiency could also occur due to defective sorting within the cells. Imerslund (1960) and Grasbeck *et al.* (1960) described a familial disorder in which megaloblastic anemia developed in children due to a failure to absorb cbl. These children contained normal amounts of intrinsic factor. Since this earlier finding, more cases in children have been reported (MacKenzie *et al.*, 1972). In these patients there was normal amounts of ileal receptor and normal absorption of fat and xylose. It is possible that the defect could be due to an intracellular transport of the newly synthesized receptor to apical plasma membranes. On the other hand, the defect might be due to the inability of intracellular acidic vesicles to release cbl bound to proteins.

A defect in the handling of TC-II-cbl complex by cultured fibroblasts from a patient has been suggested to be due to a failure in a possible transport system associated with the lysosomes (Rosenblatt *et al.*, 1985). The exact reasons for a defect in the intracellular sorting of cbl in these patients will be clearer once all the pathways in the intracellular trafficking of cbl in a normal cell is established.

Cellular entry and exit of cbl is a complex process. The story of cbl and its relation to anemia has been known for 50 years, but the slow unravelling of the complexities related to intracellular trafficking has been mainly due to a variety of reactions involved, complexity of cbl structure and the possible influence of structure on binding to intracellular proteins and hence on cellular entry and exit, and to the extremely low levels of tissue proteins which delayed their isolation. Now that all the binding proteins, and their receptors are isolated, and our knowledge in cell biology of intracellular trafficking has greatly increased the future of cbl research appears to be bright. It is hoped that a total picture on the cellular movement and turnover of cbl and its analogs will help in a clearer understanding of the pathogenesis in some forms of cbl deficiency.

## Acknowledgement

The work was supported in part by a grant from the National Institutes of Health (AM-26638).

## References

- Allen, R. H. (1975) *Prog. Hematol.*, **9**, 57.
- Allen, R. H. and Mehlman, C. S. (1973) *J. Biol. Chem.*, **248**, 3660.
- Allen, R. H., Seetharam, B., Podell, E. and Alpers, D. H. (1978a) *J. Clin. Invest.*, **61**, 47.
- Allen, R. H., Seetharam, B., Allen, N., Podell, E. and Alpers, D. H. (1978b) *J. Clin. Invest.*, **61**, 1628.
- Ashwell, G. and Morell, A. G. (1974) *Adv. Enzymol.*, **41**, 99.
- Burger, R. L., Schneider, R. H., Mehlman, C. S. and Allen, R. H. (1975) *J. Biol. Chem.*, **250**, 7707.
- Chanarin, I., Muir, M., Hughes, A. and Hoffbrand, A. V. (1978) *Br. Med. J.*, **1**, 1453.
- Ciechanover, A., Schwartz, A. L., Varsat, A. D. and Lodish, H. F. (1983) *J. Biol. Chem.*, **258**, 9681.
- Grasbeck, R., Gordin, R., Kantero, I. and Kuhlback, B. (1960) *Acta Med. Scand.*, **167**, 289.
- Immerslund, O. (1960) *Acta Paediatr. Scand.*, **119**, 1.
- Kapadia, C. R. and Donaldson, R. M. (1985) *Ann. Rev. Med.*, **36**, 98.
- Kapadia, C. R., Serfilippi, D., Voloshin, K. and Donaldson, R. M. (1983) *J. Clin. Invest.*, **71**, 440.
- Kouvonen, I. and Grasbeck, R. (1979) *Biochem. Biophys. Res. Commun.*, **115**, 358.
- Levine, J. S., Allen, R. H., Alpers, D. H. and Seetharam, B. (1984) *J. Cell Biol.*, **98**, 1111.
- MacKenzie, I. L., Donaldson, R. M., Trier, J. S. and Mathan, V. I. (1972) *New Eng. J. Med.*, **286**, 1021.
- Newmark, P., Newman, G. E. and O'Brien, J. R. P. (1970) *Arch. Biochem. Biophys.*, **141**, 121.
- Robertson, J. A. and Gallagher, N. D. (1985a) *Gastroenterology*, **88**, 908.
- Robertson, J. A. and Gallagher, N. D. (1985b) *Gastroenterology*, **88**, 1353.
- Rosenblatt, D. S., Hosack, A., Matiaszski, N. V., Cooper, B. A. and Laframboise, R. (1985) *Science*, **228**, 1319.
- Rosenberg, L. E. (1983) in *The Metabolic Basis of Inherited Disease* 5th edition (ed. J. B. Stanbury) (New York: McGraw Hill Book Company) p. 474.
- Rothenberg, S. P., Weiss, J. P. and Cotter, R. (1978) *Br. J. Hematol.*, **40**, 401.
- Schneider, R. J., Burger, R. L., Mehlman, C. S. and Allen, R. H. (1976) *J. Biol. Chem.*, **57**, 27.
- Seetharam, B., Alpers, D. H. and Allen, R. H. (1981a) *J. Biol. Chem.*, **256**, 3785.
- Seetharam, B. and Alpers, D. H. (1982) *Ann. Rev. of Nutr.*, **2**, 343.
- Seetharam, B. and Alpers, D. H. (1985) *Nutr. Rev.*, **43**, 97.
- Seetharam, B., Bagur, S. S. and Alpers, D. H. (1981b) *J. Biol. Chem.*, **256**, 9813.
- Seetharam, B., Bagur, S. S. and Alpers, D. H. (1982) *J. Biol. Chem.*, **257**, 183.
- Seetharam, B., Bakke, J. E. and Alpers, D. H. (1983) *Biochem. Biophys. Res. Commun.*, **115**, 238.
- Seetharam, B., Presti, M., Frank, B., Tiruppathi, C. and Alpers, D. H. (1985) *Am. J. Physiol.*, **248**, 326.
- Seligman, P. A. and Allen, R. H. (1978) *J. Biol. Chem.*, **253**, 1766.
- Toskes, P. P., Hansell, J., Cerda, J. and Deren, J. J. (1971) *New Eng. J. Med.*, **284**, 627.

M. K. RAY, A. MITRA, B. MUKHERJEE and G. C. CHATTERJEE\*  
Department of Biochemistry, University College of Science, 35, Ballygunge Circular Road,  
Calcutta 700 019, India

**Abstract.** Lipopolysaccharides of *Salmonella typhimurium* inhibit the activity of acetylcholinesterase *in vitro* in both synaptosomal and erythrocyte membranes. Arrhenius plots show that the transition temperatures of membrane bound acetylcholinesterase are significantly reduced in the presence of lipopolysaccharides, and the activation energies above and below transition temperature have increased with the lowering of transition temperature. These results indicate that an alteration in the fluidity of the phospholipid layer of the membranes, may be responsible for the membrane-specific effect of lipopolysaccharides on acetylcholinesterase activity.

**Keywords.** Membrane; acetylcholinesterase endotoxin; transition temperature; inhibition.

## Introduction

Gram negative bacterial toxins are broadly represented by lipopolysaccharides (LPS) which elicit their toxic effects after attachment to cell-associated extracellular receptors like the lipoglycoprotein on erythrocyte membrane (Springer and Adye, 1977). LPS administration is known to change the fluidity of hepatic plasma membrane (Conde *et al.*, 1981) and there is a close interrelationship between membrane enzyme activity and membrane fluidity (Liu *et al.*, 1983). LPS play a significant role in the development of pathophysiology of endotoxic shock at the cellular level, by decreasing membrane fluidity (Onji and Liu, 1981). Solomonson *et al.* (1976) demonstrated that alteration of plasma membrane fattyacyl composition *in vivo* results in altered properties of  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  associated with this membrane. They suggested that the fattyacyl groups as well as the polar head groups of phospholipids are important determinants of enzyme activities. Membranes undergo gel to liquid crystal-phase transition which are dependant on the fatty acid composition of the membrane. Kimelberg and Mayhew (1975) showed that the activity of certain membrane bound enzymes was increased in virally transformed cells and when this occurred, the transition temperature (TT) and the activation energy of the enzymes were altered. The apparent activation energy of most soluble enzymes is constant between 0°C and 37°C. However, mammalian membrane bound enzyme systems show a large change in activation energy over this temperature interval. Kimelberg and Papahadjopoulos (1972) suggested that changes in the activation energy of membrane bound ATPase was due to a lipid phase transition. It has been established that in most cases membrane bound enzymes show a TT in their Arrhenius plots. It has further been proved that the existence of a TT is due

To whom all correspondence should be addressed.

Abbreviations used: LPS, Lipopolysaccharides; TT, transition temperature; AchE, acetylcholinesterase; DTNB, 5,5-dithio bis (2-nitrobenzoic acid).

not to any other component of the membrane lipids surrounding and interacting with the enzyme (Grisham and Barnett, 1973). Acetylcholinesterase (AChE), one of the most important brain enzymes, is believed to be involved in impulse transmission at cholinergic synapses (Rieger and Vigne, 1976). The activity of this peripheral-extrinsic enzyme has been found to be influenced significantly by membrane phospholipids (Beauregard and Roufogalis, 1977; Sihotang, 1976). In this laboratory, studies on different aspects of LPS isolated from *Agrobacterium tumefaciens* and *Salmonella typhimurium* have been carried out (Banerjee *et al.*, 1981, 1983; Halder *et al.*, 1984; Mitra *et al.*, 1985; Ray *et al.*, 1985). In the present study however the possible changes in lipid-protein interactions of the enzyme AChE of isolated brain synaptosomal and erythrocyte membranes during the treatment with *S. typhimurium* LPS have been determined.

## Materials and methods

The chemicals used in this study were of the analytical grade, commercially available. The LPS of *S. typhimurium* was purchased from Sigma Chemical Company, St. Louis, Missouri, USA.

### *Experimental animals*

Male swiss albino mice weighing  $20 \pm 22$  g maintained on laboratory diet (Chatterjee *et al.*, 1973) and water *ad libitum* were used in the experiments.

### *Membrane preparation*

Mice brain synaptosomal membrane was prepared according to the differential method of Gray and Whittaker (1982) as modified by Bradford *et al.* (1973). Erythrocyte ghost was prepared as described by Kunimoto and Miura (1985).

### *Enzyme assay*

AChE activity was determined spectrophotometrically according to the procedure of Ellman *et al.* (1961). The final assay medium totalling 3 ml consisted of 0.29 mM 5,5-dithio bis (2-nitrobenzoic acid) (DTNB), 0.5 mM acetylthiocholine iodide and 0.05 ml of the enzyme preparation in a medium of phosphate buffer. To study the *in vitro* effect of LPS, the membrane preparation was preincubated with 50  $\mu\text{g}/\text{ml}$  of LPS at 37°C for 20 min, prior to addition of substrate. The rate of change of colour was measured at 412 nm. Assays were performed at temperatures varying from 10°–40°C at 2–5 degree intervals.

### *Protein assays*

The protein content of the enzyme preparations was estimated according to Lowry

### Estimation of activation energy

The Arrhenius equation was utilised to estimate the activation energies of the enzyme above and below the transition temperatures.

### Statistical significance

The statistical significance of differences between mean values of experimental and control was determined by Student's 't' test.

## Results

The activity of brain synaptosomal membrane bound AchE was measured at various temperatures (10°C–40°C) and it is found that the activity of the enzyme was suppressed with rise in temperature, in the presence of *S. typhimurium* LPS as compared against the control (figure 1). Figure 3 depicts the activity of erythrocyte membrane bound AchE under similar experimental conditions where inhibition in the enzyme activity with increase in temperature in the presence of LPS has also

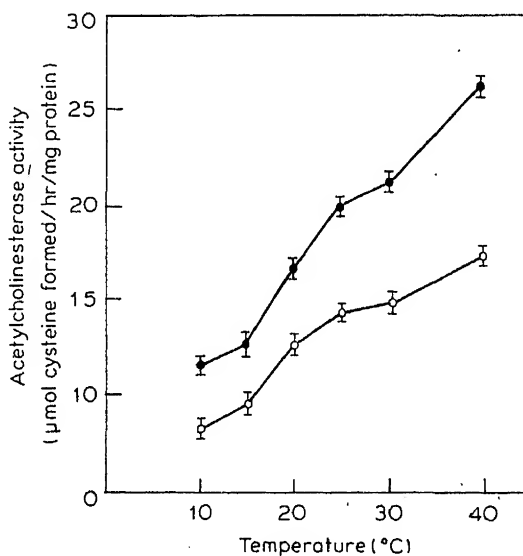


Figure 1. Effect of temperature on AchE activity in the brain synaptosomal membrane in the absence (●) and presence of 50 μg/ml (○), LPS.



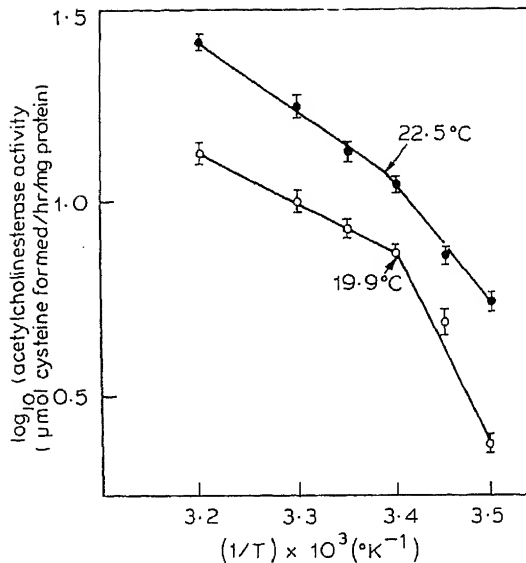


Figure 2. Arrhenius plots of AchE of brain synaptosome in the absence (●) and presence of 50  $\mu\text{g/ml}$  (○), LPS.

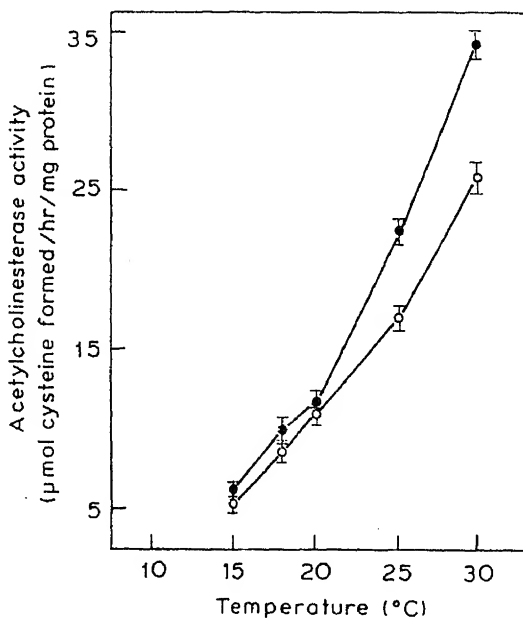
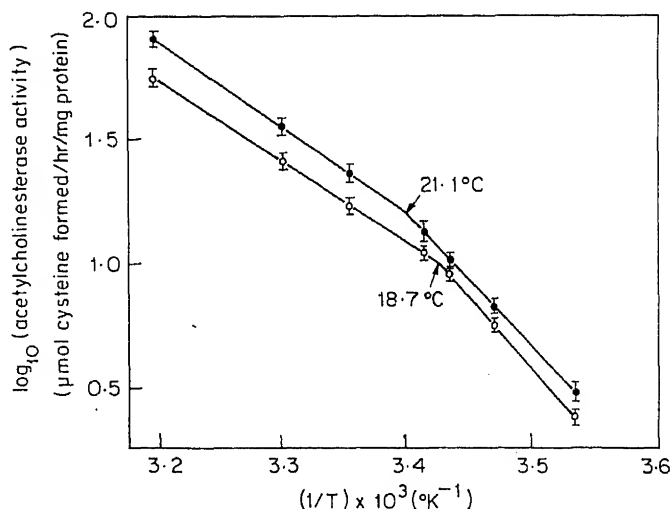


Figure 3. Effect of temperature on the erythrocyte membrane bound AchE in the absence (●) and presence of 50  $\mu\text{g/ml}$  (○), LPS.

been observed although the inhibition in the erythrocyte membrane enzyme is lower when compared with that of the synaptosomal membrane enzyme. However, the

erythrocyte membranes respectively. Examination of the plots show that the normal TT of synaptosomal AchE is 22.5°C which is lowered to 19.9°C on being treated with 50 µg/ml *S. typhimurium* LPS *in vitro*. The same amount of LPS lowered the TT of erythrocyte AchE to 18.7°C from 21.1°C.

Table 1 shows the apparent activation energy above and below the transition temperature of membrane bound AchE activity in erythrocytes and synaptosomal fraction.



**Figure 4.** Arrhenius plots of erythrocyte membrane bound AchE in the absence (●) and presence of 50 µg/ml (○), LPS.

Each point represents in the mean  $\pm$  S.D. of 5 experiments each on a different animal.

**Table 1.** *In vitro* effect of *S. typhimurium* LPS on transition temperature and apparent activation energy\* of synaptosomal and erythrocyte membrane bound AchE activity.

Membrane AchE	LPS concentration (µg/ml)	Transition temperature	Activation energy (Kcal/mol)	
			Below TT	Above TT
Synaptosome	0.0	22.5	12.937 $\pm$ 0.77	2.5 $\pm$ 0.15
	50.0	19.9	11.9 $\pm$ 0.831	4.0 $\pm$ 0.28 <sup>a</sup>
Erythrocyte	0.0	21.1	25.25 $\pm$ 1.515	15.633 $\pm$ 0.93
	50.0	18.7	28.264 $\pm$ 1.695 <sup>b</sup>	15.059 $\pm$ 0.9

\*Activation energy was calculated from the slope of the lines of figures 2 and 4.

Each result represents mean value  $\pm$  standard deviation of 5 separate determinations.

Mean value significantly different from the control group <sup>a</sup>P < 0.001, <sup>b</sup>P < 0.05.

## Discussion

AchE, like many other membrane-bound enzymes undergoes a dramatic change in apparent activation energy between 10°–40°C. Although a number of explanations

suggested as playing a major role. The present data suggest that lipid phase changes can be correlated to Arrhenius plot breaks. The work of Kimelberg (1975) and Warren *et al.* (1975) on reconstituted membrane systems containing  $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-ATPase}$  lends support to the above assumption. AchE is a peripheral extrinsic phospholipoprotein and the role of lipid especially phospholipids is vital for this enzyme activity (Mitchell and Hanahan, 1966). The lipid bilayer is believed to be the site of action of LPS and other lipophilic compounds on bio-membranes (Tanaka, 1974). Hence it is reasonable to assume that variation of lipid composition of different membranes (Solomonson *et al.*, 1976) as well as their structural organisation (Nemat-Gorgani and Meisami, 1979) may be responsible for the membrane-specific effect of LPS on AchE activity. There is indirect evidence that membrane alteration may involve a lipid change and this idea has been expressed recently by Londesberg (1980) who proposed that the higher apparent activation energy at low temperatures may result from the addition of an enthalpy component associated with a lipid phase transition around the enzyme. The sharp discontinuity in the Arrhenius plot is found at the TT which is the crystalline to liquid-crystalline phase transition temperature of membrane lipids. The TT of synaptosomal AchE is found to be  $22.5^\circ\text{C}$  and that of erythrocyte AchE is  $21.1^\circ\text{C}$ . LPS-induced lowering of TT in both membranes indicates that membrane lipid phase transition takes place at a lower temperature in the presence of LPS and this probably is brought about by an alteration in the fluidity of the phospholipid mono- and bilayer environment (Papahadjopoulos and Kimelberg 1974, Heron *et al.*, 1980).

## Acknowledgement

One of the authors (GCC) acknowledges the Council for Scientific and Industrial Research, New Delhi, for financial support.

## References

- Banerjee, D., Basu, M., Chowdhury, I. and Chatterjee, G. C. (1981) *Biochem. Biophys. Res. Commun.*, **100**, 1384.
- Banerjee, D., Basu, M. and Chatterjee, G. C. (1983) *Indian J. Biochem. Biophys.*, **20**, 154.
- Beauregard, G. and Roufogalis, B. D. (1977) *Biochem. Biophys. Res. Commun.*, **77**, 211.
- Bradford, H. F., Barnett, G. W. and Thomas, A. J. (1973) *J. Neurochem.*, **21**, 495.
- Chatterjee, G. C., Ray, R. K., Sasmal, K., Banerjee, S. K. and Mazumdar, P. K. (1973) *J. Nutr.*, **103**, 509.
- Conde, G., Garcia-Barreno, P., Munico, A. M. and Snarez, A. (1981) *FEBS Lett.*, **127**, 115.
- Ellman, G. L., Countney, D. K., Andres, V. Jr. and Featherstone, R. M. (1961) *Biochem. Pharmacol.*, **7**, 88.
- Gray, E. G. and Whittaker, V. P. (1982) *J. Anat.*, **96**, 79.
- Grisham, C. M. and Barnett, R. E. (1973) *Biochemistry, Easton*, **12**, 2635.
- Haldar, S., Basu, A., Chakraborty, K., Addya, S., Santra, M. and Chatterjee, G. C. (1984) *Indian J. Exp. Biol.*, **22**, 123.
- Heron, D. S., Shinitzky, M., Hershkowitz, M. and Sammel, D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7463.
- Kimelberg, H. K. (1975) *Biochim. Biophys. Acta*, **413**, 143.
- Kimelberg, H. K. and Mayhew, E. (1975) *J. Biol. Chem.*, **250**, 100.
- Kimelberg, H. K. and Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta*, **282**, 277.
- Kunimoto, M. and Miura, T. (1985) *Biochim. Biophys. Acta*, **816**, 37.
- Liu, M. S., Ghosh, S. and Yang, Y. (1983) *Life Sci.*, **33**, 1995.

- owry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- itchell, C. D. and Hanahan, D. J. (1966) *Biochemistry, Easton*, **5**, 51.
- ittra, A., Ray, M. K. and Chatterjee, G. C. (1985) *IRCS Med. Sci.*, **13**, 968.
- emat-Gorgani, M. and Meisami, E. J. (1979) *J. Neurochem.*, **32**, 1027.
- nji, T. and Liu, M. S. (1981) *Circ. Shock*, **8**, 403.
- apahadjopoulos, D. and Kimelberg, H. K. (1974) *Prog. Surf. Sci.*, **4**, 141.
- ay, M. K., Mitra, A., Tiwari, R. K., Mukherjee, B. and Chatterjee, G. C. (1985) *Indian J. Exp. Biol.*, **23**, 116.
- ieger, F. and Vigne, M. (1976) *J. Neurochem.*, **27**, 121.
- hotang, K. (1976) *Eur. J. Biochem.*, **63**, 519.
- olomonson, L. P., Liepkalns, V. A. and Spector, A. A. (1976) *Biochemistry*, **15**, 892.
- pringer, G. F. and Adye, J. C. (1977) in *Microbiology* (ed. D. Schlessinger) (Washington D. C.: Am. Soc. Microbiol.) p. 326.
- anaka, R. (1974) in *Reviews in Neuroscience* (eds S. Ehrenpreis and I. J. Kopin) (New York: Raven Press) vol. 1, p. 181.
- arren, G. B., Metcalfe, J. C., Lee, A. G. and Birdsall, N. J. M. (1975) *FEBS Lett.*, **50**, 261.



V. SITARAMAM\* and M. K. JANARDANA SARMA

National Institute of Nutrition, Jamai Osmania P.O., Hyderabad 500 007, India

**Abstract.** Isotonic requirements for synaptosomes were shown to vary with the concentration of sucrose or mannitol in the isolation medium, as well as with their differential permeability to polyols and ions. The technique of enzyme osmometry, which permits quantitation of the osmotic integrity in a heterogeneous population, was used to define the osmotic requirements for synaptosomes and myelosomes in a variety of ionic and non-electrolyte media. Important differences, observed in the rank order of permeability of synaptosomal and myelosomal membranes to electrolyte media, were consistent with the known channel density/electrical activity of the corresponding plasma membranes.

**Keywords.** Enzymes osmometry; turbidimetry; synaptosomes; myelosomes; lactate dehydrogenase; osmolytes.

## Introduction

Physical integrity of synaptosomes is an essential prerequisite for transport studies *in vitro* and awaits a clear definition. Basically, 3 types of definitive studies were reported in literature that question the inherent assumption of controlled physical integrity of synaptosomes and therefore the validity of such uptake studies in synaptosomes: (i) Sperk and Baldessarini (1977) showed that synaptosomes exhibit pronounced leakage of internal lactate dehydrogenase, when incubated in Krebs-Ringer medium, unless stabilized by sucrose; (ii) Adamvizi and Marchbanks (1983) claimed that osmotic 'disruption is never complete and although resealing does occur, the contribution of residual synaptosomes is difficult to distinguish' in the experimental protocols for uptake studies (*cf.* Kanner and Sharon, 1978; Kanner, 1978; Roskoski *et al.*, 1981); (iii) dramatic changes in the permeability characteristics of biological membranes such as synaptosomes, mitochondria, peroxisomes (Sitaramam and Sarma, 1981a,b; Sambasivarao and Sitaramam, 1983), myelosomes (Sarma and Sitaramam, 1982), lysosomes (Reijngoud and Tager, 1977), hepatocytes (Sainsbury *et al.*, 1979) were shown to occur during centrifugation, leading to equilibration of small molecular weight substances such as sucrose, with consequent altered isotonic requirements. Thus, a methodological reappraisal of the integrity of synaptosomes has become truly complex, not only in relation to preparative methods, but also in terms of osmotic requirements for studies on transport.

These considerations have prompted us to investigate and delineate the physical criteria of integrity of synaptosomes, which is long over due in neurochemical research. The still novel technique of enzyme osmometry is derived from a quantitative interpretation of the activity profiles of occluded enzymes, based on the robust physical theory of osmosis. In this paper, we document briefly the methodology and

---

\*To whom all correspondence should be addressed.

Abbreviations used: LDHase, Lactate dehydrogenase; B.P., break point.

applications of enzyme osmometry as relevant to synaptosomal integrity, for the first time. This technique also aided in the elucidation of the structure of osmotically active myelin particles, the myelosomes (Sarma and Sitaramam, 1982) which was independently confirmed subsequently (Pereyra and Braun, 1983). The technique of enzyme osmometry was used also to demonstrate the different osmotic requirements for the integrity of synaptosomes and myelosomes.

## Materials and methods

### *Materials*

Sucrose, sodium pyruvate, mannitol,  $\beta$ -NADH, Tris and Triton X-100 were obtained from Sigma Chemicals Co., St. Louis, Missouri, USA. All other reagents were of analytical grade.

### *Isolation of synaptosomes*

Synaptosomes from rat neocortex as well as motor cortex of Rhesus monkeys (*Macaca mullata*) were isolated by method of deRobertis *et al.* (1962). The crude mitochondrial fraction ( $P_2$ ) was resolved into 5 fractions labelled as A–E., corresponding to the interface of the discontinuous sucrose gradient: A (0.32–0.8 M), B (0.8–1.0 M), C (1.0–1.2 M), D (1.2–1.4 M) and E (as a pellet in 1.4 M). Aliquots of fractions C and D were diluted slowly, with ice-cold, glass distilled water under constant stirring, to a final sucrose concentration of 0.32 M sucrose. The particles were washed twice with 0.32 M sucrose at 0.4°C by centrifugation at 10,000 *g* to yield fractions  $C_L$  and  $D_L$  respectively (*cf.* Sitaramam and Sarma, 1981a).

### *Preparation of 'mannitol' synaptosomes*

Whole brains from 4 rats were homogenized in 0.3 M mannitol medium (without EDTA or buffer to avoid undue clumping of particles) and were processed according to the method of Kanner and Sharon (1978) in a discontinuous ficoll gradient with 0.3 M mannitol throughout. The 8–12% ficoll interface were collected and diluted with 3 volumes of 0.3 M mannitol and centrifuged again to obtain 'mannitol' synaptosomes, free of ficoll, finally suspended in 0.3 M mannitol.

### *Isolation of myelosomes*

Osmotically active myelin particles were obtained from primate cortical white matter as described earlier (Sarma and Sitaramam, 1982). Crude mitochondrial fraction ( $P_2$  (M)) and purified myelin (fraction A (M) at 0.32–0.8 M sucrose interface) were harvested by an isolation technique identical to that of deRobertis *et al.* (1962).

Relative specific activity of lactate dehydrogenase (LDHase, L-lactate:NAD<sup>+</sup>-oxidoreductase, EC 1.1.1.27), monoamine oxidase, acetyl and butyryl-thiocholine-terases,  $\beta$ -galactosidase, 2'3'-cyclic nucleotide 3'-phosphohydrolase were determined in each fraction to confirm that the isolation procedures match those reported in literature (*cf.* deRobertis *et al.*, 1962; Jones, 1974). The enriched fractions of the rat neocortex consisted of: (i), Myelin; (ii), nonspecific membranes; (iii), synaptosomes; (iv), synaptosomes with contamination of mitochondria and lysosomes; (v), mitochondria and lysosomes. In the subfractionation of motor cortex of primate brain, results were similar with minor quantitative differences (data not given).

## General

Protein was estimated by a modified method of Lowry in the presence of sodium dodecyl sulphate (Markwell *et al.*, 1978). LDHase and turbidimetry assays were carried out using Gilford Spectrophotometer with automatic cuvette positioner and recorder (Sitaramam and Sarma, 1981a). Buffer and substrate concentrations in the assays were reduced so as to minimize their influence on the osmolality of the external medium. Initial velocities were determined for specific activity calculations; reaction velocities were first order with regard to synaptosomal protein.

*Enzyme osmometric methodology* (Sitaramam and Sarma, 1981a,b, Sambasivarao and Sitaramam, 1983)

The first premise in the technique of enzyme osmometry is that the activity of an occluded enzyme, E, under initial velocity conditions, is

$$\begin{aligned} J_r &= K A_o / (1 + K/P) \\ \text{and, } A_i &= A_o / (1 + K/P) \\ \text{since, } J_r &= K A_i, \end{aligned} \quad (1)$$

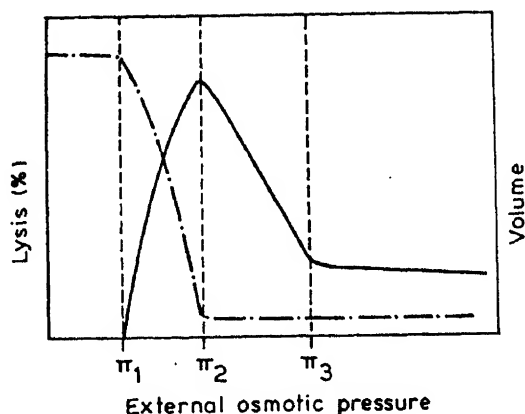
where  $K$  is the rate constant of the enzyme and  $P$ , the permeability to the external substrate,  $A_o^*$ . The second premise is that the osmotic behaviour of a population of

The equation implies an equality that  $P(A_o - A_i) = K A_i$  which is valid only for an occluded enzyme. An occluded enzyme, typified by LDHase, is defined as that whose activity is stimulated significantly, at constant  $A_o$ , when the membrane barrier is removed by means such as solubilization (detergents) or disruption (freeze-thaw etc.), which do not significantly interfere with the activity of the occluded enzyme. Presence of occluded activity, therefore, necessarily implies  $A_o > A_i > A_c$ , where  $A_c$  is the minimal substrate concentration corresponding to  $V_{\max} \delta$ , where  $\delta$  is an immeasurably small decrement in activity (since  $V_{\max}$  requires an infinite substrate concentration). This excludes the possibility of inequality of the kind  $(A_o - A_i) > K A_i$  due to  $A_i \geq A_c$ . Similarly, initial velocity conditions (which is a rigorous equilibrium assumption) itself excludes the inequality of the kind,  $P(A_o - A_i) < K A_i$  since  $J_r = K A_i$ .

In principle, one may argue that non-osmotic leakage of enzyme, cofactors or inhibitors could affect



critical limits of external osmolality as depicted in figure 1. The osmotic phenomena broadly fall into two categories. In one, the measurements are associated with lysis,



**Figure 1.** Osmotic profiles in membranous particles. The volume of a population of particles varies with external osmotic pressure linearly within two critical limits,  $\pi_2$  and  $\pi_3$ , such that the isotonicity corresponds to  $(\pi_2 + \pi_3)/2$ . The onset of lysis corresponds to the maximal volume of the particles at  $\pi_2$ . Total lysis of the population is achieved at a critical external osmotic pressure,  $\pi_1$ , corresponding to 'zero' internal (i.e., occluded) volume of the particles. An evaluation of the points of inter-section of the individual regression lines yields the critical external osmotic pressures  $\pi_1$ - $\pi_3$ . The Y-coordinate values corresponding to the break-points at  $\pi_2$  and  $\pi_1$  yield '0' and 100% lysis (or 100 and 0% integrity, respectively).

activity and even contribute to inequality. This is simply overcome by the fact that break-point is independent of activity, since it represents the critical external osmolality at which the activity exhibits a marked non-linearity. Break-point represents a true non-linearity (i.e., discontinuity) because: (i) it is independent of transformations on either or both axes, and (ii) no single polynomial, that too of a high order, can account for all the experimentally observed profiles in a variety of systems and (iii) the break-point remains virtually the same even when a significant amount of the occluded enzyme is released and the particles resealed, as in fractions  $P_2$ ,  $C_L$  and  $D_L$ . Non-osmotic mechanisms of leakage (of either enzyme or endogenous modulators of activity), if any, should exhibit only a monotonic profile as a function of osmolality. Any true non-linearity would arise only due to an osmotic mechanism and would only serve to reinforce the break-point measurement. It would be a truism to state that any or a set of linear operators would not impart non-linearity to a response. However, the signal-to-noise ratio ( $K/P$ ) may be affected. Thus, absence of an osmometric profile need not be sufficient evidence for the absence of a barrier, while its presence is the most conclusive evidence for osmotic behaviour.

Since there could always be some external contamination of the enzyme, a more complete expression would be,

$$J_r = KA_o \left[ \frac{E_b}{1 + K/P} + E_f \right],$$

$$E_{\text{total}} = E_b + E_f$$

where  $E_b$  is the total occluded enzyme and  $E_f$  is the external free enzyme. Another kind of inequality can arise in the osmotic space, if either  $K$  or  $P$  vary with the volume of the particles. This is excluded by critically showing that break-point of osmometry is identical to that of osmolysis, as shown in the case of LDHase. Existence of a break-point in the non-lytic domain has interesting implications on the nature of transport (vide discussion). In any event, the kinetic and physical assumptions inherent to the osmometric analysis are eminently testable.

es, one corresponding to the onset and the other corresponding to the completion of lysis. The derivative of the lysis profile would be a direct measure of the variance associated with a population of particles, it being a cumulative integral of the osmotic heterogeneity of the entire population. The volume profile, the second category, which is the signal-averaged response of a population, does not yield readily information on the heterogeneity of the population and is also characterized by the presence of two discontinuities, corresponding to the limits of linear osmotic response of a population of particles. The discontinuities can be evaluated by fitting the individual slopes (*i.e.*,  $y = m_1x + c_1$  and  $y = m_2x + c_2$ ) across each discontinuity corresponding to  $\pi_1$ ,  $\pi_2$  and  $\pi_3$  in figure 1) to regression lines, such that the point of intersection for any pair of slopes, may be obtained as the break-point concentration on x-axis; B.P. =  $(c_2 - c_1)/(m_1 - m_2)$ . Lysis can be monitored either by release of occluded enzymes into cell-free supernatants (osmolysis), or, on direct suspension of the particles in the assay medium adjusted to various tonicities by a chosen osmolyte and directly monitoring the activity (osmometry). Given the condition that  $P \ll K$  (to ensure good signal-to-ratio), under initial velocity conditions, osmolysis and osmometry would yield identical break-points for the onset of lysis (*i.e.*, expression of occluded activity). If  $P$  is also a function of volume of the particle, a break-point to the right of lysis would be obtained in osmometric profiles. A break-point to the left of osmolytic profiles is forbidden. Since volume of the particles can also be monitored by rate of change in absorbance (turbidimetry) (Sambasivarao and Sitaramam, 1983; Sarma and Sitaramam, 1982), one can verify these assumptions in a turbid preparation such as myelosomes (though not readily in synaptosomes) that the break-point of osmometry of turbidimetric data (*i.e.*, volume) always falls to the right of osmometry of occluded enzymes with impermeable substrates or osmolysis.

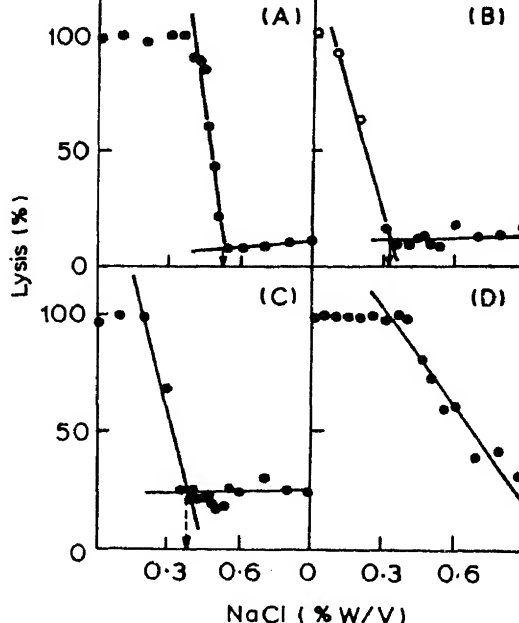
Figure 2 illustrates these principles using erythrocyte osmolysis profiles. Addition of as little as 50 mM Tris HCl (82 mOs/kg) was seen to cause a measurable shift in the break-point of onset of lysis by  $\sim 62$  mOs/kg. Similarly, loss of internal  $K^+$  on addition of valinomycin should lower the instantaneous osmotic pressure exerted by internal  $K^+$  and therefore offer protection against hypotonic lysis by a left-ward shift in the break-point, while enhanced permeability to ions (particularly external  $Na^+$ ) by addition of gramicidin S should make the cells more susceptible to lysis, as indicated by a marked right-ward shift of the break-point (figure 2C and D) (*cf.* Sambasivarao and Sitaramam, 1985; Sambasivarao *et al.*, 1986).

These results demonstrate the unique capability of the osmometric technique to measure the reflection coefficients to external and internal osmolytes and variations thereof. The instantaneous osmotic pressure required for constant tonicity by a permeable external solute, at time,  $t = 1$  is,

$$\pi_{s(t=1)} = \pi_{s(t=0)} + RT \cdot ds \int_{t=0}^{t=1} dt, \quad (2)$$

such that the shift in break-point for an erythrocyte population was,

$$\Delta \text{B.P.} = RT \cdot J_{Na^+} \int_0^t dt - RT \cdot J_{K^+} \int_0^t dt, \quad (3)$$



**Figure 2.** Erythrocyte osmolytic curves. (A), Control osmolytic profile in varying NaCl solutions with 3.3 mM Tris-HCl, pH 7.4; (B), osmolytic profile in the presence of 50 mM Tris-HCl, pH 7.4; (C), osmolytic profile as in A, in the presence of  $10^{-6}$  M valinomycin in the osmolytic media; (D), osmolytic profiles as in A, in the presence of  $10^{-5}$  M gramicidin S.

Break-point analysis was carried out as described in the text for both the onset and the completion of lysis, wherever measurable. The figures illustrate only the evaluation of the break-point of the onset of lysis. The break-points (B.P.) (expressed as % NaCl) for the onset of lysis were: (A), 0.53%, (B), 0.33%, (C), 0.387% and (D), not measurable (>0.9%). B.P. Indicated by a dashed arrow.

where  $J_{Na^+}$  and  $J_{K^+}$  represent the influx and efflux  $Na^+$  and  $K^+$  respectively\* (Sambasivarao *et al.*, 1986).

The second unique feature of the technique osmometry of is that the confidence interval associated with the break-point obeys an F distribution (Rao, 1967) such that the

\*More generally,

$$\Delta B.P. = \frac{RT}{V_c} \left\{ \begin{matrix} J_p - J_q \\ 1 \rightarrow 2 & 2 \rightarrow 1 \end{matrix} \right\}$$

where  $V_c$  is the critical, constant volume corresponding to the break-point (Os/kg),  $J$ , the fluxes, *i.e.*, the influx (1→2) of the external ( $p$ ) and the efflux (2→1) of the internal ( $q$ ) osmolytes, in time,  $t$ , since  $\pi = RTC$ , where  $C$  is the concentration. Since,

$$J = L\Delta\mu + J_{act}$$

where  $L$  is the Onsager matrix of phenomenological coefficients (including permeability/diffusion coefficients, transport capacity etc.);  $\Delta\mu$  the passive forces (concentration, electrical forces) and  $J_{act}$ , the contribution of active mechanisms, one can experimentally derive the relationship between the break-point and the net permeation of any given species of osmolyte, by appropriate design. For a more detailed discussion

tions for the technique of osmometry have been published earlier (Sitaramam and Sarma, 1981b; Sambasivarao and Sitaramam, 1983). A detailed statistical theory (since no ready methodology exists) will be published elsewhere. Briefly, the negative slope is referred to as the determinant slope, whose coefficient of correlation is a measure of the fit of the data to a rectangular distribution, *i.e.*, equal probability of osmotic susceptibility for the entire population, determined by its range, the break-points. The high sensitivity of break-point analysis to systematic error requires that the data on the same day be compared with adequate internal controls rather than data from different preparations on different days.

It should be noted that the variance associated with the break-point (*i.e.*, intrinsic to each profile) results in a coefficient of variation of magnitude less than 5%. However, the profiles, when assayed under identical conditions on the same preparation yield break-points which are almost indistinguishable (*i.e.*, they differ within 1%). As the profiles are susceptible to systematic errors likely to occur over different days, it is natural to expect larger variation in the data base and hence, also the break-points, which indeed was the case. For instance, when the break-points of 6 different  $P_2$  fractions assayed on different days were compared, the break-points varied within 10% of coefficient of variation. Using a single day preparation, we could show that the variations in the specific activity of occluded LDHase activity (due to its variable removal by lysis) as in  $P_2$ ,  $C_L$  and  $D_L$  fractions, did not contribute significantly to a change in break-points. Therefore, one should not pool the data collected over different days to arrive at estimates of reliability or robustness of the break-points by mere calculation of standard deviation for the break-points.

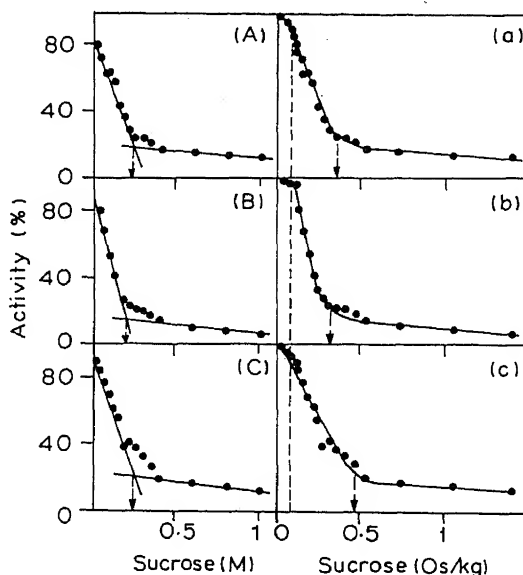
### *Osmolality vs concentration*

The osmolality of various assay media were routinely determined using a Wescor vapour pressure osmometer. In osmometric as well as osmolytic experiments, break-point analysis was routinely performed, using concentration of external osmolyte rather than osmolality, since the break-points represent a robust measurement of material properties, relatively independent of transformations (*i.e.*, molarity, molality, osmotic pressure (bar)) on the primary variable, *viz.* the osmotic pressure of the external medium.

## **Results**

The experimental results reported here have 4 specific objectives, *i.e.*, (i) to illustrate the efficacy of the technique of osmometry to achieve a clear definition of the physical integrity synaptosomes and myelosomes; (ii) to resolve the controversy whether 100% lysis of synaptosomes can indeed be achieved, as prelude to studies on transport with defined internal milieu; (iii) to identify standardized protocols in the investigation of integrity of synaptosomes; and lastly, (iv) to investigate the differences between the osmotic behaviour of synaptosomes and myelosomes. The definition of integrity was throughout restricted to physical integrity in terms of matrix space enclosed by a limiting membrane (which alone is measurable and is applicable to all cells and organelles), avoiding more qualitative notions such as metabolic integrity.

Phenomenological homology (*i.e.*, identical break-points) of LDHase osmometry (*i.e.*, direct measurement of activity of LDHase in particulate suspensions without phase separation) and osmolysis (*i.e.*, release of the enzyme into supernatants, as in the case of hemoglobin) was documented by us earlier (Sitaramam and Sarma, 1981a). This equivalence emerges as a necessary consequence of the impermeability of the exogenous NADH to the cell membrane. LDHase osmometry in various synaptosomal fractions isolated in a constant 0.32 M sucrose media was consistent with the theoretical predictions (Sitaramam and Sarma, 1981a,b) thus: (i) a marked negative slope bounded by a discontinuity (break-point) was seen in the osmometric profiles of various fractions (also in figure 3 A-C); (ii) the physical basis of LDHase osmometry was identical to that of osmolysis, since the break-points were identical; (iii) the break-point of LDHase osmometry matched not only the sucrose content within the organelle, but also the ambient sucrose concentration of each fraction



**Figure 3.** LDHase osmometry of synaptosomal fractions,  $P_2$  (A,a),  $C_L$  (B,b) and  $D_L$  (C,c), isolated in 0.32 M sucrose. The osmometric profiles in A-C represent the activity of the enzyme plotted as a function of external sucrose concentration, assayed in the presence of 50 mM Tris-HCl. The osmometric profiles in a-c represent the activity plotted as a function of osmolality of the assay medium; the osmolality was lowered further by decreasing Tris-HCl concentration from 50–10 mM (*i.e.*, below zero sucrose concentration indicated by a vertical dashed line). Break-point analysis was carried out in all cases as described in the text. B.P. (indicated by a dashed arrow) values were: (A), 0.26 M; (B), 0.27 M; (C), 0.27 M, external sucrose; and (a), 0.35; (b), 0.3; (c), 0.37 Os/kg. The regression lines of break-point analysis were depicted in A-C; a-c represent visual fit of the sigmoidal osmometric profiles to facilitate comparison with figures 1 and 2. 100% activity was taken as that obtained in 10 mM Tris-HCl, for reasons discussed in the text. Specific activities of total LDHase in these fractions were comparable to those reported by Sitaramam and Sarma (1981a), for rat

olated by density gradient centrifugation at 0–4°C; (iv) the break-point was insensitive to the permeability to the external solute, such that the osmometric profiles yielded the rank order of permeability, *i.e.*, sucrose < Na<sup>+</sup> < K<sup>+</sup>.

Methodological investigations in a wide variety of systems led to the following rules in obtaining osmometric profiles of good statistical quality. (i) Osmotic pressure of the external medium should be predominantly contributed by a single osmolyte of choice. (ii) The substrate should have very low permeability and the occluded enzyme high activity, to ensure good signal-to-noise ratio in break-point analysis. (iii) Initial velocity conditions should be strictly adhered to. (iv) Velocity should be first order with regard to protein in the assay medium at all osmolalities. (v) The external osmolyte should exhibit only a monotonic profile of inhibition of the solubilized enzyme with a slope much smaller than that obtained in enzyme osmometry.

These studies posed specific cautions with regard to the criteria of integrity of synaptosomes. Release of macromolecules such as LDHase or hemoglobin is the only reliable measure of the integrity of membranes, but not that of ions such as K<sup>+</sup> because: (i) large changes in break-point in K<sup>+</sup> media (compared to sucrose media) indicating rapid permeation of K<sup>+</sup>; (ii) the reported prelytic increase in K<sup>+</sup> permeability in erythrocytes (Seeman *et al.*, 1969); (iii) osmotic dependence of K<sup>+</sup> exchangers (Garlid, 1980); (iv) enhanced permeability to K<sup>+</sup> in synaptosomes (Sitaramam and Sarma, 1981a) and erythrocytes (Davson and Danielli, 1938) during centrifugation and (v) mediation of K<sup>+</sup> influx depending on the metabolic state *via* Na<sup>+</sup>, K<sup>+</sup> ATPase. Attempts to quantify the integrity of synaptosomes in terms of K<sup>+</sup> content (Adam-Vizi and Marchbanks, 1983) would be unfructuous, since marked changes in K<sup>+</sup> content could occur without membrane disruption, hypotonic or otherwise. Even the release of LDHase should be interpreted with caution, as shown below.

The specific question with regard to release of LDHase was whether it yields evidence for 100% lysis of synaptosomes. 100% lysis would be indicated by (i) ability to assay all the occluded activity in hypotonic media, the total activity judged from Triton X-100 solubilized synaptosomes and (ii) the true sigmoidal shape of the osmolytic/osmometric profile as in the case of erythrocytes, such that maximal lysis corresponds to 100% lysis. Osmometric profiles in figure 3A–C, of fractions *P*<sub>2</sub>, *C*<sub>L</sub> and *D*<sub>L</sub> (all of which were isolated in 0.32 M sucrose) show that even at zero sucrose concentration, 10–15% synaptosomes remain intact, as also pointed out by Adam-Vizi and Marchbanks (1983). Since the usually recommended buffer concentration, *i.e.*, 50 mM Tris-HCl (Marchbanks, 1967; Whittaker and Barker, 1972), alone would contribute to a finite (measured) osmotic pressure of 82 mOsm/kg, we varied Tris-HCl from 10–50 mM in the absence of sucrose, as an extension of the hypotonic domain and measured the LDHase activity as a function of true osmolality. Indeed the osmometric profiles yielded not only a sigmoidal profile as in the case of erythrocytes but also further release of LDHase. The activity of LDHase on solubilization by Triton X-100 was actually lower by 5–10% than that obtained in 50 mM Tris-HCl buffer. Thus, the activity of LDHase in these fractions in 10 mM Tris-HCl buffer approximated the total activity rather than that with Triton, which was marginally inhibitory. The break-points in the osmolality scale (figure 3a–c) were larger than those in the molarity scale (figure 3A–C), as expected of the contri-

finite, though relatively minor, variations in the true break-point concentration. In figure 3, plots in both scales were given specifically to illustrate the similarity regardless of the scale chosen in osmometric studies. Plotting in the osmolality scale also revealed the close proximity of the break-point to the isotonic medium of 0.32 M sucrose (350 mOs/kg), showing that the nearly spherical synaptosomal populations exhibit very little capacity for volume increase. Even the biconcave erythrocytes cannot withstand the surface area expansion beyond 3% without lysis (Linderkamp and Meiselman, 1982). The Y-intercept at the break-point corresponds to the osmotically insensitive basal activity, the external LDHase contamination, by 8–10%. It may be recalled that solubilized LDHase exhibits only a monotonic inhibition in the presence of sucrose (Sitaramam and Sarma, 1981a).

#### *Extent of lysis in synaptosomes prepared in mannitol media*

Kanner and Sharon (1978) prepared synaptosomes in 0.3 M mannitol, lysed in 5 mM Tris-HCl and resealed in 0.1 M potassium phosphate buffer, after storage and freeze-thaw in 0.32 M sucrose. Adam-Vizi and Marchbanks (1983) questioned the wisdom of such a complex procedure since no attempt was made by Kanner to quantitate the contribution of non-lysed synaptosomes.

In order to investigate the extent of lysis in 'mannitol'-synaptosomes, synaptosomes were prepared by the method of Kanner and Sharon (1978) and the osmometry of LDHase was carried out with sucrose as an external osmolyte (figure 4). The break-point shifted to left by 25% compared to 0.32 M sucrose synaptosomes (correcting for the differences in the osmolality of the different isolation media), suggesting that the internal mannitol was more permeable than the external sucrose.

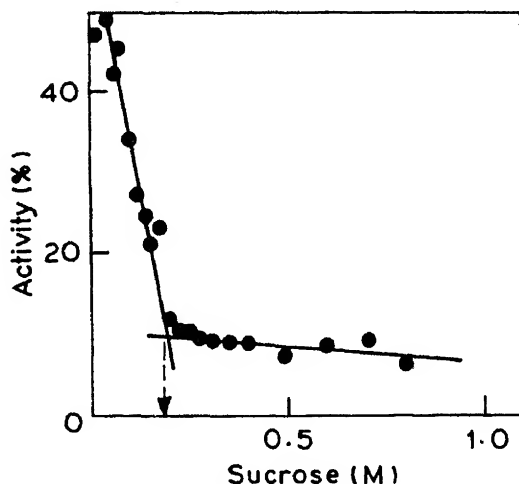


Figure 4. LDHase osmometry for mannitol synaptosomes. Synaptosomes were prepared in 0.3 M mannitol, as described in the text. Total activity, assayed in the presence of Triton X-100 was taken as 100%; the activity assayed in the presence of 50 mM Tris-HCl buffer, pH 7.4, was plotted as a function of the external sucrose concentration. B.P. represented by

A corresponding shift in the negative slope to left resulted in a lysis of only 70–75% of the synaptosomes even in water, or ~50% lysis in 50 mM Tris-HCl. The criticism levied by Adam-Vizi and Marchbanks (1983) was therefore valid in so far as the lysis step in 5 mM Tris-HCl was concerned. However, Kanner employed a further step of freeze-thaw which would be expected to disrupt all the synaptosomes. Doubt persists since possible lysis during transport assays was not monitored and the absence of a critical control study of uptake at 0°C at all time points tested was conspicuous.

#### Isotonic requirements for synaptosomal fractions

The recommended procedure for an evaluation of the integrity of synaptosomes was to assay for LDHase in synaptosomes in 50 mM Tris-HCl buffer and to obtain fold stimulation of the activity on subsequent addition of Triton X-100 during the assay itself (Marchbanks, 1967; Whittaker and Barker, 1972). The magnitude of fold stimulation depends directly on (i) the integrity of synaptosomes, (ii) the amount of LDHase within, (iii) the relative impermeability of the substrate and inversely on (iv) the amount of external contamination of free LDHase, provided that adequate controls exist for absence of NADH oxidation by other enzymatic means and interference due to changes in turbidity on addition of Triton X-100. Since the assay of LDHase as in 50 mM Tris-HCl buffer results in ~90% lysis (with consequent reduction in fold stimulation by Triton), we investigated fold stimulation of LDHase activity by Triton, using different fractions of rat brain as well as primate cortical synaptosomes (which were subsequently needed for comparison with myelosomes) obtained by the method of deRobertis *et al.* (1962), as a preliminary step to arrive at their isotonic requirements. Figure 5a shows that fold stimulation in 50 mM Tris-HCl buffer (*i.e.*, without sucrose) was barely 2–5 fold in various fractions of synaptosomes from primate motor cortex. Though specific activity of LDHase was highest in

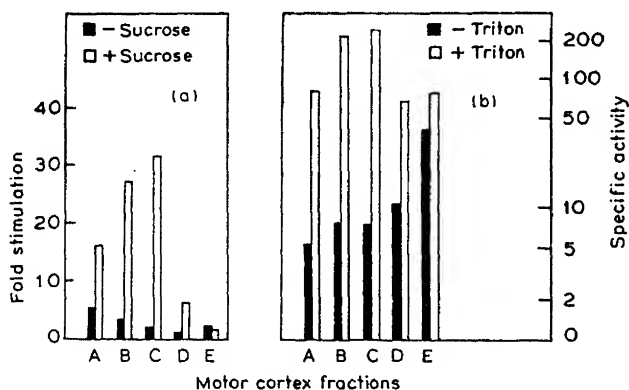


Figure 5. Occluded LDHase activity profiles in various deRobertis *et al.* (1962) fractions isolated from the  $P_2$  fraction of primate motor cortex. LDHase activity was determined in the presence of 50 mM Tris-HCl, pH 7.4, with and without 0.32 M sucrose. Fold stimulation



fraction C (figure 5b), fold stimulation by Triton was the least in synaptosomal fractions C and D (figure 5a). Contamination with external LDHase was difficult to visualize in these fractions, since density gradient centrifugation would remove much of the external contamination. On the other hand, inclusion of 0.32 M sucrose in the assay medium resulted in marked improvement of fold stimulation, particularly in fraction C, though not in fraction D. Clearly, fold stimulation would be an ideal marker for integrity and the choice of the assay medium, provided that the choice of the medium is applicable to all the fractions. Figure 5b shows the specific activity of all the fractions in 0.32 M sucrose with 50 mM Tris-HCl buffer with and without Triton. The total activity of LDHase recovered from P<sub>2</sub> was mainly in fractions C, D and A, in that order (data not given). The total specific activity (*i.e.*, in the presence of Triton) was maximal in fraction C, but not in the absence of Triton. In fact, when assayed in 0.32 sucrose media, the basal activity of LDHase in these fractions progressively increased corresponding to sucrose concentration actually ambient during the density gradient isolation of these fractions. This anomalous behaviour could be reconciled with only if the isotonic requirements vary with the ambient sucrose concentration during centrifugal isolation, thereby affecting integrity of various fractions differentially, when assayed at a constant osmolality (Sitaramam and Sarma, 1981a).

Since equilibration of sucrose across biological membranes during centrifugal isolation was unequivocally established (Sitaramam and Sarma, 1981a,b; Sambasivarao and Sitaramam, 1983), we compared fold stimulation of various fractions by varying the sucrose concentration in the assay medium corresponding to that of the medium of isolation for each fraction. Data in table 1 compared LDHase activity (thereby, fold stimulation) in the deRobertis *et al.* (1962) fractions of rat neocortex. Clearly,

**Table 1.** Occluded LDHase activity in deRobertis *et al.* (1962) fractions obtained from rat neocortex.

Fraction	Isotonic <sup>a</sup> sucrose, M	LDHase specific <sup>b</sup> activity (total)	LDHase specific activity <sup>c</sup>		
			50 mM Tris-HCl buffer only	0.32 M + isotonic sucrose	+ isotonic sucrose
P <sub>2</sub>	0.32	0.509	0.417 (82)	0.059 (11.6)	0.059 (11.6)
A	0.5	0.284	0.136 (48)	0.063 (22)	0.0457 (16)
B	0.9	0.44	0.387 (88)	0.156 (35)	0.080 (18)
C	1.1	1.04	0.758 (73)	0.066 (6.3)	0.030 (2.8)
D	1.3	0.401	0.340 (85)	0.151 (37.6)	0.037 (9.2)
E	1.4	0.601	0.674 (112)	0.34 (56.5)	0.053 (8.7)

<sup>a</sup>Isotonic sucrose corresponds to the average sucrose concentration of the density interface at which each fraction is isolated, since sucrose was known to equilibrate with the interior during centrifugal isolation of synaptosomes (*vide text*).

<sup>b</sup>Total activity was assayed on prior solubilization in 0.1% (v/v) Triton X-100.

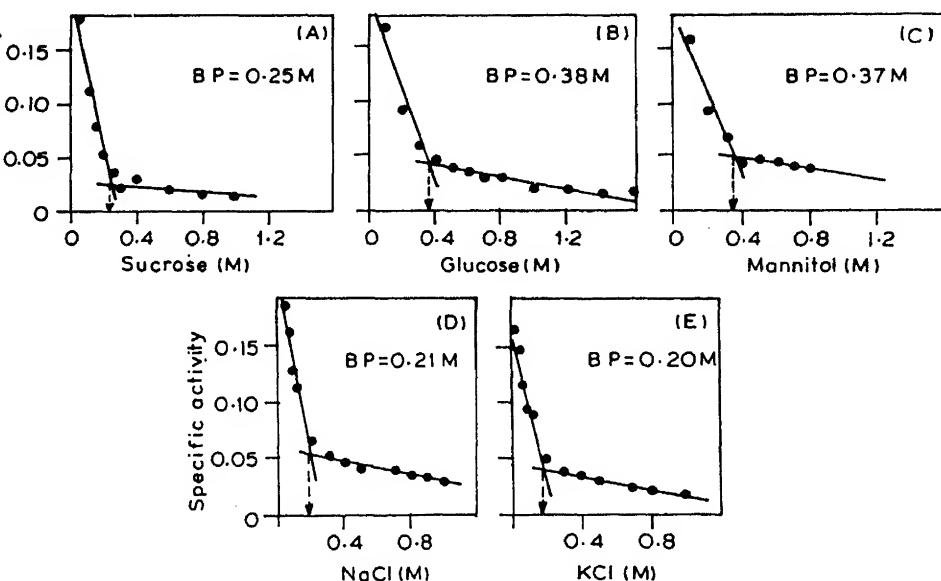
<sup>c</sup>Aliquots of each fraction were assayed in duplicate in media of different composition, as indicated. Numbers in parentheses represent % activity, taking total activity(b) as 100. Fold stimulation (*a/b*) can

ness of whether the source of synaptosomes was rat or primate cortex, or even the primate retina (data not given).

The significance of these experiments is two fold: (i) Since an experimental protocol such as in table 1 permits rapid screening of media for the required tonicity, detailed LDHase osmometric profiles, otherwise tedious and time consuming, may be substituted by such a protocol, (ii) the practice of diluting subcellular fractions from sucrose density gradients directly into the so called 'isotonic' media (0.32 M sucrose for synaptosomes or 0.25 M sucrose for mitochondria) would be logically absurd and would result in uncontrolled bursting and resealing, however slowly diluted. Oxidative phosphorylation in mitochondria, a sensitive index of their integrity, cannot be demonstrated in mitochondria isolated in sucrose density gradients and diluted into 0.25 M sucrose (*cf.* Sambasivarao and Sitaramam, 1983).

*Rank order of permeability to electrolytes and non-electrolytes in synaptosomes and myelosomes*

Uncontrolled lysis and resealing of synaptosomes during transport assays can be preempted only if the rank order of permeability to electrolytes and non-electrolytes is critically assessed for a given preparation of synaptosomes. Data in figure 6 shows detailed osmometric profiles in fraction  $C_L$  of primate motor cortex. Since NaCl and



**Figure 6.** LDHase osmometry in synaptosomal fraction,  $C_L$ , from primate motor cortex, in sucrose (A), glucose (B), mannitol (C), NaCl (D), KCl (E), media in the presence of 50 mM Tris-HCl buffer. Break-point analysis was carried out as described in the text (B.P., indicated by a dashed arrow). The osmometric profiles were obtained in a single experiment. Specific activity was defined as in figure 5.

data clearly shows that the rank order of permeability (=rank order of break-points) was sucrose < glucose  $\approx$  mannitol < NaCl  $\approx$  KCl. Thus, as predicted by eq. (2), the technique of osmometry appears to be a reliable method to assess the reflection coefficients to external solutes.

Onset of lysis should correspond to the maximal volume of the particles, *i.e.*, the break-point of volume changes should be larger than that of lysis (figure 1). Classical measurements of reflection coefficients rely on the monitoring of volume changes by light scatter methodology (Goldstein and Solomon, 1961). Carvalho and Carvalho (1979) and Kamino and Inouye (1975) attempted light scatter studies in synaptosomes. Experience with the application of light scatter measurements in erythrocytes showed that it would be hazardous to measure volume changes with concomitant lysis by light scatter (Seeman *et al.*, 1969; Goldstein and Solomon, 1961; Mlekoday *et al.*, 1983; Passow, 1964). The osmometric analysis of turbidimetry circumvents this limitation since it could be critically compared with osmolytic profiles and break-points. Assessment of permeability by the osmometric technique is less direct than the classical method of reflection coefficient measurements. It was therefore necessary to assess volume changes, atleast in a comparable particle, by osmometric methodology. The requirement for such a particle would be: (i) origin from the same tissue/cell, (ii) the presence of occluded LDHase activity and (iii) high capacity of the particle for light scatter. Myelosomes would be the obvious choice, since these particles, as we described earlier (Sarma and Sitaramam, 1982), would also be the choice control material for uptake studies on synaptosomes.

Data in table 2 summarizes the break-point analysis of detailed osmometric profiles of LDHase and turbidimetry in NaCl, KCl, sucrose, glucose and mannitol media in various fractions of synaptosomes from rat and primate cortex as well as myelosomes isolated from the white matter of primate brain. A major advantage in such a comparison was that, while marginal contamination of myelin material in synaptosomes is unavoidable, preparation of myelosomes from pure white matter guaranteed the lack of contamination by synaptosomes. The rapidity of changes in

**Table 2.** Rank order of permeability to polyols and electrolytes in synaptosomal and myelin (axonal) plasma membranes, as measured by LDHase osmometry and osmometry linked to turbidimetry.

Osmolyte	Rat neocortex <sup>a</sup>		Cortical white matter (primate)			Motor cortex <sup>b</sup> (primate)
	C	D	P <sub>2</sub> (M)	A (M)		C <sub>L</sub>
	LDHase	LDHase	Turbidimetry	Turbidimetry	LDHase	LDHase
Sucrose	0.49	0.86	0.36	0.61	0.39	0.245
Glucose	N.D.	N.D.	—	—	> 0.40 <sup>c</sup>	0.378
Mannitol	N.D.	N.D.	—	—	> 0.40 <sup>c</sup>	0.368
NaCl	0.46	0.59	0.133	0.24	0.12	0.207
KCl	0.51	0.8	0.115	0.1	0.102	0.202

<sup>a</sup>Data from Sitaramam and Sarma, 1981a.

<sup>b</sup>Data from figure 5. N.D. not done. —Data omitted since statistical criteria not satisfied.

<sup>c</sup>Exact break-point could not be determined (*vide text*). Various fractions were isolated at the interfaces of sucrose solutions. C<sub>1</sub> (1.0–1.2 M), D<sub>1</sub> (1.2–1.4 M) and C<sub>L</sub> (0.32 M) represent synaptosomal fractions; P<sub>2</sub> (M)

hand, the high permeability to these polyols also resulted a shift in the break-point of LDHase osmometry to right, to the extent that the break-point could not be evaluated (*cf.* figure 2D). The data clearly showed that glucose and mannitol were more permeable than sucrose. Since neither mannitol nor sucrose would be expected to share the glucose transporter (they being foreign and inert) (Sitaramam and Sarma, 1981a; Sambasivarao and Sitaramam, 1983), the enhanced permeability to mannitol requires adequate explanation. In the case of electrolytes, synaptosomes exhibited greater permeability, while, myelosomes exhibited lesser permeability by osmometry of LDHase as well as turbidimetry. The data confirms the validity of LDHase osmometry, since turbidimetric profiles yielded consistently larger break-points than the corresponding profiles of LDHase. The differences in the rank order of permeability between myelosomes and synaptosomes for electrolytes supports our earlier contention that the myelosomes offer a unique control material to study what is specific to the plasma membrane of nerve endings.

## Discussion

The experiments reported here demonstrate unequivocally the importance of the technique of osmometry in the quantitative delineation of the physical integrity of synaptosomes and myelosomes. The underlying statistical theory, based on an assumption of rectangular distribution of osmotic susceptibility of particles and its validation by least square regression fit of the determinant slope permit conclusions valid for the entire population. An evaluation of shifts in break-points offers a volume-independent method of evaluation of fluxes of osmolytes, overcoming the inherent limitation of centrifugal methodology for phase separation. The studies also confirm the results of Sperk and Baldessarini (1977) with regard to the interference due to coincident lysis during transport assays due to permeability to external  $\text{Na}^+$  and  $\text{K}^+$ . Lysis of synaptosomes leading to 100% activity in 10 mM Tris-HCl indicated that the synaptosomes exhibit an osmolytic hole similar to that observed in erythrocytes (Lieber and Steck, 1982). Presence of 100% activity during LDHase osmometry indicated that the closure of the osmolytic hole was achieved by subsequent step of centrifugation for reisolation of synaptosomes after osmolysis, which again imparts osmotic behaviour to the LDHase remaining within. Thus 100% lysis can only be evaluated by direct osmometric methodology and not after attempts at phase separation in conventional lysis experiments.

Failure to observe good osmometric profiles in literature invariably resulted from complex assay conditions and the use of synaptosomes from sucrose density gradients, without reisolation in sucrose media of constant osmolality. Enhanced heterogeneity (*i.e.*, larger range of the determinant slope) could be shown to be a result of variable internal osmolyte content as well as presence of permeant osmolytes externally. On the other hand, strict observance of the methodological rules of osmometry invariably led to distinct break-points of high statistical quality, provided that, (i) synaptosomes were isolated in large diameter centrifuge tubes in swing-out buckets (to avoid wall effects), (ii) synaptosomes were repeatedly pelleted in the same sucrose medium and (iii) external osmolality was largely due to a single osmolyte of low permeability.

The differential osmotic behaviour of synaptosomes and myelosomes is of considerable importance for several reasons: (i) Enhanced permeability of the membranes of both these particles to mannitol, compared to sucrose, was in accordance with the observations of Villegas *et al.* (1966) on the enhanced permeability of the electrically active squid axonal plasma membrane to non-electrolytes. Since permeation of non-electrolytes, such as mannitol, would be thermodynamically forbidden (Sitaramam and Sarma, 1981a,b; Sambasivarao and Sitaramam, 1985), it is important to investigate the effect of membrane potential on non-electrolyte conductance to investigate possible alterations in the fine structure of these membranes due to electrical activity. (ii) Enhanced permeability of the synaptosomal membrane to  $\text{Na}^+$  and  $\text{K}^+$  as opposed to the myelosomal membrane would be consistent with the known electrical activity and channel density for these ions in these membranes ( $25/\mu\text{m}^2$  for the axonal plasma membrane which is relatively inert, as opposed to a density of  $10,000/\mu\text{m}^2$  for the comparable electrically active internodal membrane (Ritchie and Rogart, 1977). Thermal fluctuations in these channels would account for the observed fluxes of these ions, which are indeed large, considering the measurable variations in the instantaneous osmotic pressure exerted by these ions, as evidenced by osmometric studies.

Our studies, therefore, critically question certain assumptions inherent to transport studies. Foremost is the assumption that transport across the membrane is independent of the physical state of the membrane, *i.e.*, osmotic stretch. In a variety of enzyme systems, transporters and even channels, the activity,  $A$ , of the protein was shown to obey the relationship,

$$A = A_{\text{max}} - \bar{K}\pi, \quad (4)$$

where  $\bar{K}$  is a newly described, empirical, elastic constant relating the activity of such hydrophobic proteins to the external osmotic pressure,  $\pi$  (Sambasivarao and Sitaramam, 1985; Sambasivarao *et al.*, 1986; Garlid, 1980; Kaiser, 1982; Rao and Sitaramam, 1984; Takanaka and O'Brien, 1975). From an osmometric point of view, existence of  $\bar{K}$  relates to a relaxation of the assumption inherent to present studies on LDHase that  $P$  (permeability to the substrate) is independent of the volume of the particle. The data of Kanner and Sharon (1978) indeed suggests that L-glutamate transport activity in synaptosomes exhibits  $\bar{K}$ . Another related assumption in transport studies relates to the thermodynamic interpretation of force-flux relationships, which assume a constant volume of the matrix space (Marchbanks, 1975), which would not be valid in the face of a permeable electrolyte\*. Studies are in progress to delineate the importance of such physical interactions in transport across synaptosomal and myelosomal membranes.

## References

- Adam-Vizi, V. and Marchbanks, R. M. (1983) *J. Neurochem.*, **41**, 780.  
 Bunow, B. (1978a) *J. Theor. Biol.*, **75**, 51.

\*The dimensionless quantity,  $K/P$  (eq. 1), is one form of Thiele modulus (see Bunow, 1978a,b for a more detailed exposition), used by chemical engineers to measure the strength of transport effects on chemical reactions. Existence of  $\bar{K}$  for the rate constant of catalysis( $K$ ) and/or permeability or transport activity( $P$ )

- Carvalho, C. A. M. and Carvalho, A. P. (1979) *J. Neurochem.*, **33**, 309.
- Davson, H. and Danielli, J. F. (1938) *Biochem. J.*, **32**, 991.
- deRobertis, E., de Iraldi, A. P., Arnaiz, G. R. L. and Salganicoff, L. (1962) *J. Neurochem.*, **9**, 23.
- Garlid, K. D. (1980) *J. Biol. Chem.*, **255**, 11273.
- Goldstein, D. A. and Solomon, A. K. (1961) *J. Gen. Physiol.*, **44**, 1.
- Hanes, D. G. (1974) *Synapse and synaptosomes* (London: Chapman and Hall).
- Kaiser, W. M. (1982) *Planta*, **154**, 538.
- Kamino, K. and Inouye, A. (1975) *J. Colloid Interface Sci.*, **53**, 275.
- Kanner, B. I. and Sharon, I. (1978) *Biochemistry*, **17**, 3949.
- Kanner, B. I. (1978) *Biochemistry*, **17**, 1207.
- Lieber, M. R. and Steck, T. L. (1982) *J. Biol. Chem.*, **257**, 11651.
- Linderkamp, O. and Meiselman, H. J. (1982) *Blood*, **59**, 1121.
- Marchbanks, R. M. (1967) *Biochem. J.*, **104**, 148.
- Marchbanks, R. M. (1975) *J. Neurochem.*, **25**, 463.
- Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. E. (1978) *Anal. Biochem.*, **87**, 206.
- Mlekoday, H. J., Moore, R. and Levitt, D. G. (1983) *J. Gen. Physiol.*, **81**, 213.
- Passow, H. (1964) in *The Red Blood Cell* (eds C. Bishop and D. M. Surgenor), (New York: Academic Press) p. 71.
- Pereyra, P. M. and Braun, P. E. (1983) *J. Neurochem.*, **41**, 957.
- Rao, C. R. (1967) *Linear statistical inference and its applications* (New York: John Wiley and Sons) p. 251.
- Rao, N. M. and Sitaramam, V. (1984) *3rd European Bio-energetics Conference*, Short reports, (Hannover: ICSU Press) vol. 3B, p. 555.
- Reijngoud, D. J. and Tager, J. M. (1977) *Biochim. Biophys. Acta*, **472**, 419.
- Ritchie, J. M. and Rogart, R. B. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 211.
- Roskoski, R. Jr., Rauch, N. and Roskoski, L. M. (1981) *Arch. Biochem. Biophys.*, **207**, 407.
- Sainsbury, G. M., Stubbs, M., Hems, R. and Krebs, H. A. (1979) *Biochem. J.*, **180**, 685.
- Sambasivarao, D. and Sitaramam, V. (1983) *Biochim. Biophys. Acta*, **722**, 256.
- Sambasivarao, D. and Sitaramam, V. (1985) *Biochim. Biophys. Acta*, **806**, 195.
- Sambasivarao, D., Rao, N. M. and Sitaramam, V. (1986) *Biochim. Biophys. Acta*, **857**, 48.
- Sarma, M. K. J. and Sitaramam, V. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 362.
- Seeman, P., Sanks, T., Argent, W. and Kwant, W. O. (1969) *Biochim. Biophys. Acta*, **183**, 476.
- Sitaramam, V. and Sarma, M. K. J. (1981a) *Proc. Natl. Acad. Sci. USA*, **78**, 3441.
- Sitaramam, V. and Sarma, M. K. J. (1981b) *J. Theor. Biol.*, **90**, 317.
- Spierk, G. and Baldessarini, R. J. (1977) *J. Neurochem.*, **28**, 1403.
- Takanaka, K. and O'Brien, P. J. (1975) *Arch. Biochem. Biophys.*, **169**, 428.
- Villegas, R., Villegas, G. M., Blei, M., Herrara, F. C. and Villegas, J. (1966) *J. Gen. Physiol.*, **50**, 43.
- Whittaker, V. P. and Barker, L. A. (1972) *Methods Neurochem.*, **2**, 1.



## Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblasts cells induced by glucocorticoids<sup>§</sup>

HASI R. DAS†, ROBERT BARATZ\* and DELANO V. YOUNG\*\*

CSIR Centre for Biochemicals, V. P. Chest Institute Building, Delhi University Campus, Delhi 110 007, India

\*Department of Anatomy, Boston University School of Medicine, 80 E. Concord Street, Boston, Massachusetts 02118, USA

\*\*Damon Biotech Inc., 119, Fourth Avenue, Needham Heights, Massachusetts 02215, USA

**Abstract.** A number of morphological changes accompany the  $G_2$  blockage caused by glucocorticosteroids in simian virus 40-transformed 3T3 mouse fibroblasts cells. Under phase contrast microscopy dexamethasone-treated cells have darkened and raised nuclear regions with 'lines' running over their cytoplasmic areas. They are more resistant to trypsinization and smaller in volume. Since inhibitors of DNA and protein synthesis prevent this 'glucocorticoid morphology', the 'darkening' may be due to the accumulation of macromolecules within  $G_2$ -blocked cells and the induction of a protein(s) may be needed for the morphological changes. Colchicine and cytochalasin B do not bring about the glucocorticoid morphology, suggesting that it is not due to a general de-polymerization of microtubules or microfilaments. With scanning electron microscopy treated cells show a great reduction in the amount and a re-organization of microvilli and microplicae. Granules of lead precipitate at the periphery are also clearly evident in transmission electron micrographs. These observations reveal profound morphological alterations, including cell surface changes, induced by glucocorticosteroids.

**Keywords.** Cell surface; morphological changes; growth inhibition; simian virus; 3T3 mouse fibroblast cells; glucocorticoids.

### Introduction

Glucocorticosteroids are modulators of cell growth both *in vitro* and *in vivo*. For some cells glucocorticoids have been observed to enhance cellular growth (Hayashi *et al.*, 1978; Holley and Kiernan, 1974) perhaps by facilitating  $G_1$  progression into S phase, while for others growth is inhibited (Fodge and Rubin, 1975; Grasso *et al.*, 1978; Pratt and Aronow 1966; Sarah *et al.*, 1986). In the latter case, apparent blocks in both  $G_1$  (Fodge and Rubin, 1975) and  $G_2/M$  (Grasso *et al.*, 1978) have been described. Despite the effort of many laboratories in this area the precise biochemical mechanisms by which these steroid hormones either stimulate or prevent growth are unknown.

The proliferation of simian virus 40-transformed 3T3 mouse fibroblasts (SV3T3) is severely inhibited by glucocorticosteroids (Young and Dean, 1980). Typically, gluco-

<sup>§</sup>This work was carried out at the Department of Chemistry, Boston University, 685, Commonwealth Avenue, Boston, Massachusetts 02215, USA.



corticoids block cell division by 20–45 h after their addition to cells cultivated in low calf serum (0.30% v/v) Dulbecco's Modified Eagle's medium (DME) supplemented with biotin (DMEB). The growth inhibition is completely reversible, specific for glucocorticoids and no other steroid types, seen with steroid concentrations ranging from 5 ng/ml (for triamcinolone acetonide) to 50 ng/ml (for dexamethasone), and is not due to increased cytotoxicity. Recent experiments (Das *et al.*, 1983) have indicated that after cell division is almost entirely blocked, DNA, RNA, and protein synthesis continue, resulting in the overaccumulation of DNA and protein in glucocorticoid-treated cells. This has led to the conclusion of a blockage in late G<sub>2</sub> or early mitosis. Of potential significance to the problem of neoplastic transformation is the observation that in untransformed 3T3 cells glucocorticoids are generally considered to be growth stimulants (Hayashi *et al.*, 1978; Holley and Kiernan, 1974).

Accompanying the growth inhibition in SV3T3 and other cells are a number of striking morphological changes. Some of the SV3T3 structural alterations are distinctly different from those reported for other glucocorticoid-inhibited cells (Berliner and Gerschenson, 1975; Freund *et al.*, 1975; Furcht *et al.*, 1979; Holbrook *et al.*, 1980). The objectives of this article are to describe these morphological changes in detail for SV3T3 cells (as seen in both optical and electron microscopy), to offer partial explanations, and to attempt to relate some of them with the growth inhibition induced by glucocorticoid action.

## Materials and methods

### Chemicals

The tissue culture chemical supplies, including the powdered DME and calf serum, were obtained from Grand Island Biological Company, Grand Island, New York, USA. Dexamethasone, colchicine,  $\beta$  and  $\gamma$  lumicolchicine, cycloheximide, and adenine-9- $\beta$ -D-arabinofuranoside (Ara-A) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Cytochalasin B was bought from both Sigma Chemical Co. and Aldrich Chemical Co., Milwaukee, Wisconsin, USA and the products of both companies were found to yield identical results. Biotin was provided by Calbiochem-Behring Corp., San Diego, California, USA.

### Cell culture

The Swiss 3T3 and SV3T3 cells (obtained originally from Robert Holley) were maintained in DME (including 4500 mg/l glucose and 110 mg/l sodium pyruvate) supplemented with 10% (v/v) calf serum, 227 units/ml penicillin, and 33.6  $\mu$ g/ml streptomycin as described previously (Young and Dean, 1980). Cells were discarded at 6 week intervals and new cells recovered from frozen stocks. The stock cells were found to be free of mycoplasma contamination by [<sup>3</sup>H]-thymidine autoradiography.

In all experiments cells were trypsinized with 1:250 trypsin (Gibco) and inoculated in 0.3% (v/v) calf serum DMEB (DME with 100 ng/ml biotin, 10 ng/ml

dishes in a total, combined volume which did not exceed one-tenth the original medium volume. The medium was then not replaced for the entire duration of the experiment. In some cases subsequent additions of various inhibitors were made hours or even days later as explicitly described in the figure legends.

### *Scanning electron microscopy*

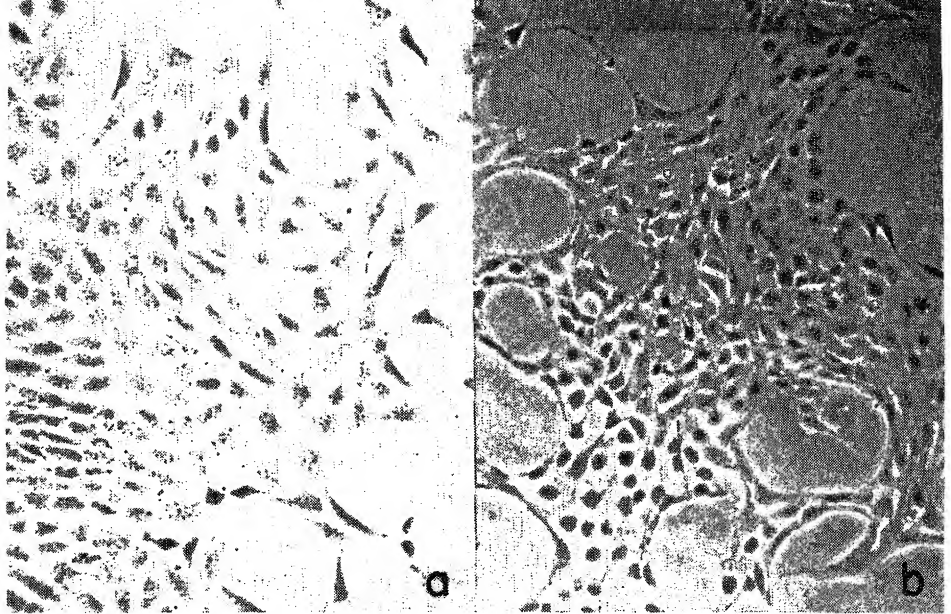
Cells grown on glass coverslips in 10 cm dishes were fixed by immersion of the coverslips in a fixative consisting of 2% paraformaldehyde and 1.6% glutaraldehyde in 0.06 M s-collidine buffer, pH 7.3, at room temperature for a minimum of 2 h. The cover slips were next loaded into a silicon rubber grid holder (Baratz, 1976) and remained there through all subsequent processing. The specimens were washed in 0.1 M s-collidine buffer, pH 7.3, exposed to 1% osmium tetroxide in the same buffer for 30 min and, following a brief rinse in this buffer, were dehydrated using graded acetone solutions (70, 95 and 100%). The samples were then quickly placed in an acetone filled boat in a pressure-bomb type critical point dryer and dried using liquid CO<sub>2</sub> as the exchange medium. The specimens were then affixed to aluminium stabs with silver paint and a film of gold-palladium was evaporated on the surface using an argon ion-sputtering device (final film thickness ~10 nm). The processed samples were then transferred to the stage of the scanning electron microscope (ISI model #60) and examined at 30 kv. Photographs were made with Polaroid type 55 P/N film.

### *Transmission electron microscopy*

Cells were fixed on their tissue culture dishes by exposure for 1 h with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. They were then rinsed with 6.4% sucrose in this buffer and treated with 2.0% osmium tetroxide dissolved in the sodium phosphate buffer. After another rinse in 6.4% sucrose-0.1 M sodium phosphate, pH 7.4, the specimens were dehydrated with ethanol and then stained with either uranyl acetate (Stempak and Ward, 1964) and lead citrate (Reynolds, 1963) or uranyl acetate and phosphotungstic acid (Hall *et al.*, 1945). The uranyl acetate-lead citrate staining followed two slightly different protocols: either 3% methanolic, ethanolic uranyl acetate for 12 min followed by 0.3% lead citrate for 2 min or 2% methanolic uranyl acetate for 20 min then 1% lead citrate for 7 min. Either process produced identical results. The uranyl acetate-phosphotungstic acid staining involved exposure to 2% uranyl acetate for 5 min and 1% phosphotungstic acid for 10 min. The actual electron micrographs were taken with a Phillips EM 300 at the Electron Microscope Service Facility of the Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

## **Results**

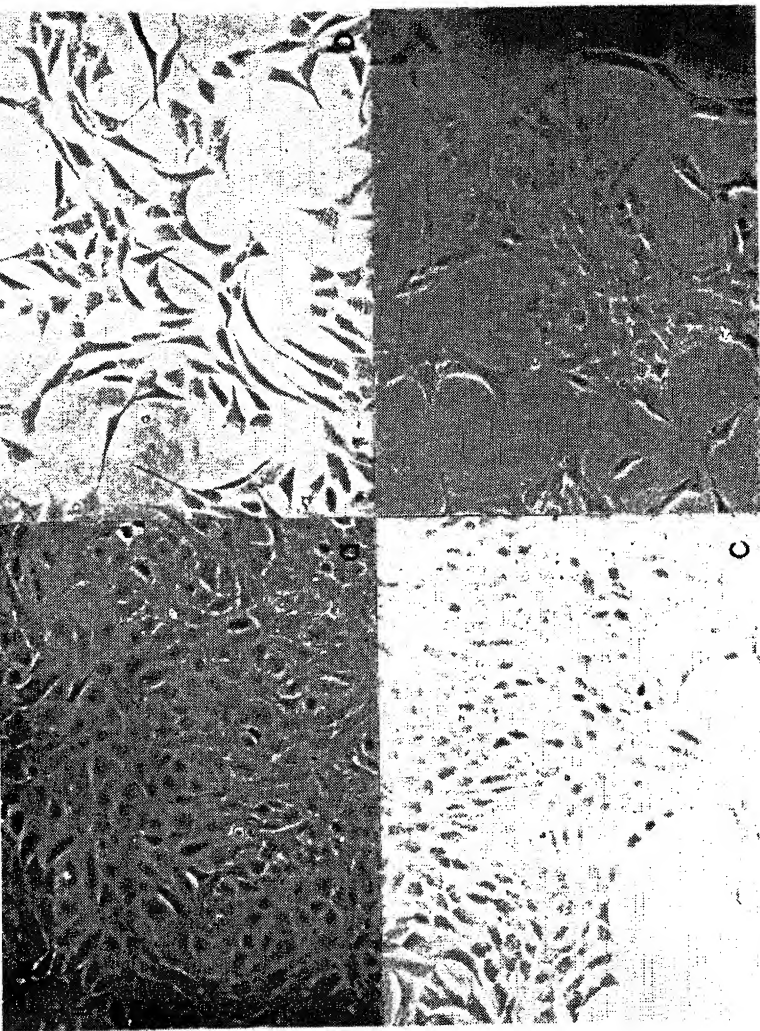
### *Structural changes as seen in phase contrast optical microscopy*



**Figure 1.** Dexamethasone-induced morphological changes in SV3T3 cells as seen in optical microscopy. The cells were inoculated into low serum DMEB (a) without or (b) with dexamethasone as described in 'materials and methods'. Photographs were taken (a) 2 days (*i.e.*, ~48 h) and (b) 4 days later. Typically, on day 1 (or 24 h later) these changes are apparent in only a few cells, by day 2 they appear in greater than 70% of the cells and by day 4 in over 90%. In these and in most of the optical photographs (figures 1-3) the culture media were removed and replaced with phosphate buffered saline just prior to photography. Phase contrast,  $\times 150$ .

of the majority (~70-80%) of the cells in culture, as viewed under phase contrast, has also undergone dramatic changes (figure 1a,b). Dexamethasone-treated cells have prominent, darkened or condensed centers; this darkening or 'thickening' under phase contrast denotes an increase in optical interference and suggests a build-up of cellular material. On the other hand, the cytoplasmic regions appear much lighter, and one can occasionally discern 'lines' running over their surfaces. The combination of these morphological changes gives the impression of cells shrinking or contracting from the dish's surface, especially at the edges of colonies, but in reality, the steroid-treated cells are probably more tightly attached to the substratum since their trypsinization is more difficult. Upon suspension the cells are found to be considerably smaller in volume (by about one-third to one-half) as observed both in the light microscope and *via* light scattering measurements employing a flow microfluorometer. These morphological changes do not occur in glucocorticoid-treated 3T3 cells.

Some of these morphological alterations can be prevented and even apparently reversed by the use of inhibitors of macromolecular synthesis. For instance, if Ara-A, an inhibitor of DNA synthesis, is added together with dexamethasone, the intense darkening of the cell center does not occur in a



**Figure 2.** Prevention and partial reversal of glucocorticoid changes by inhibitors of DNA and protein synthesis. Dexamethasone and the inhibitor of protein synthesis, cycloheximide, were added to SV3T3 cells and the photographs taken according to the following schedules: (a), Dexamethasone and Ara-A ( $20 \mu\text{g}/\text{ml}$ ) added simultaneously, 2 days later; (b), dexamethasone and cycloheximide ( $1 \mu\text{g}/\text{ml}$ ) added together, photograph taken 2 days later; (c), cycloheximide added after dexamethasone, photograph taken 1 day later; (d), cycloheximide ( $20 \mu\text{g}/\text{ml}$ ) added 3 days after dexamethasone, photograph taken next day (data not shown).  $\times 150$ .

addition simultaneously with dexamethasone prevents the development of the 'glucocorticoid morphology' (figure 2b); even the cell shape is virtually indistinguishable from normal. Cycloheximide can also be added one day after dexamethasone, a time before major morphological changes are evident, with the retention of normal morphology. This is seen in figure 2c in which cycloheximide was added 22 h after dexamethasone, and the micrograph taken two days later. In experiments in which cycloheximide was introduced 3 days after dexamethasone, at which time the glucocorticoid-induced changes are expressed in nearly all the cells, a partial restoration of the normal morphology was observed by the next day (figure 2d). The return to normality is not instantaneous so that even at 24 h some cells still possess the glucocorticoid morphology.

It is possible to interpret these results by attributing some of the darkening in the cell's center to the accumulation of macromolecules (for example DNA and protein) within a smaller volume, *i.e.* denser cells, which is prevented by the inhibition of DNA and protein synthesis. This interpretation is supported by biochemical measurements which show that dexamethasone-treated cells have a higher than normal content of DNA and protein (Das *et al.*, 1983). However, the results of the experiments involving the delayed addition of cycloheximide do not so easily conform to this interpretation unless one assumes protein turnover on a large scale. An alternative explanation, although not mutually exclusive with the above, might hold that, while some of the darkening may be due to the accumulation of macromolecules, the continued synthesis of a glucocorticoid-induced protein(s) is required for the initiation and maintenance of the morphological changes (Das *et al.*, 1983).

Since alterations in cell shape are often mediated by the organizational state of microtubules and microfilaments, the possible involvement of these structural components in the glucocorticoid-induced changes was tested. Neither colchicine nor cytochalasin B is capable of eliciting the same morphological alterations as dexamethasone (figure 3a,b). In general, both agents cause the appearance of multinucleated cells, the nuclei of which, unlike the glucocorticoid case, seem clear or transparent. The cells are larger and more broadened (*i.e.*, with greater cytoplasmic volume) than with dexamethasone treatment. Cells in colchicine concentrations of 0.04  $\mu\text{g/ml}$  and above sometimes have spike-like projections on their periphery radiating towards the substratum. Furthermore, while dexamethasone is not toxic over a broad concentration range (Young and Dean, 1980), both colchicine and cytochalasin B begin to kill these cells within a few days in the concentrations used here. It should be noted that lumicolchicine, an analog which does not bind to tubulin, does not evoke any of the colchicine effects. All of these results taken together demonstrate that the glucocorticoid morphology is not brought about by a large and generalized increase in the de-polymerization of microtubules or microfilaments.

Nevertheless, these observations do not exclude a rearrangement (*i.e.*, a transient de-polymerization-re-polymerization) of microtubules and/or microfilaments by glucocorticoids. For instance, it has been proposed (Berliner and Gerschenson, 1975) that in RLC-GAI epithelial cells glucocorticoids may elicit the cell spreading seen in that case by acting through microfilaments, since the cell spreading is blocked by cytochalasin B. When dexamethasone is combined with colchicine or cytochalasin B in the SV3T3 system, the appearance of the culture no longer closely resembles that

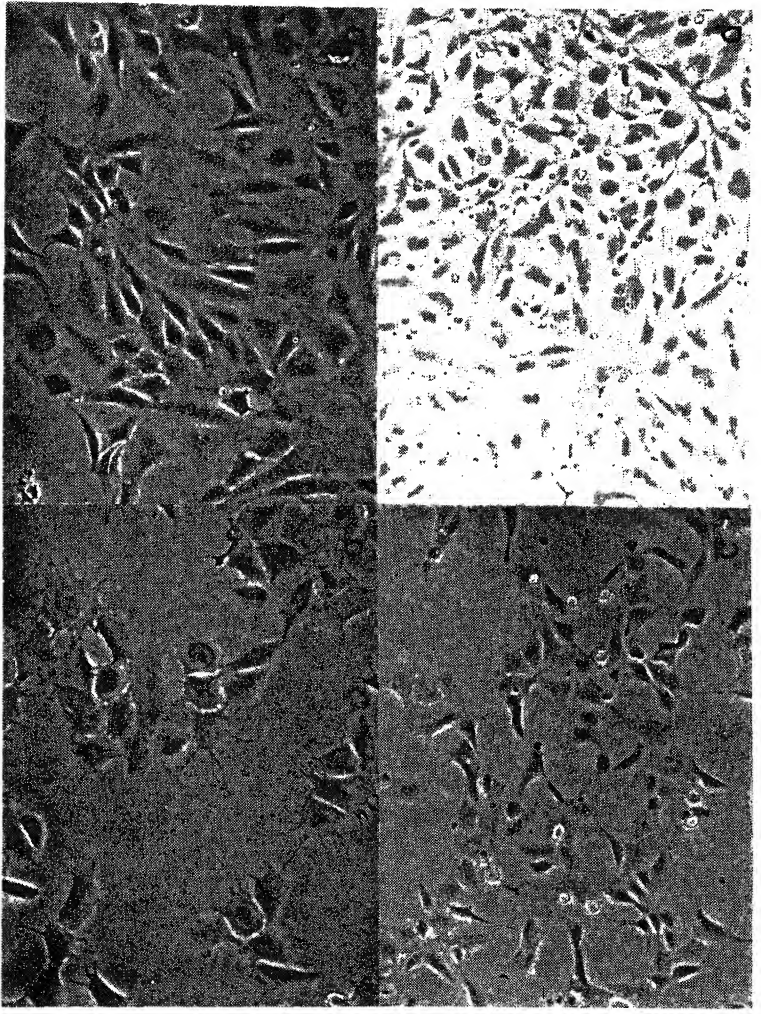


Figure 3. SV3T3 morphology in the presence of colchicine or cytochalasin B without or with dexamethasone. Cells were grown in the presence of these agents or combinations of these agents and photographs taken as follows: (a), colchicine at  $0.1 \mu\text{g/ml}$ , photograph taken on day 2; (b), cytochalasin B ( $2 \mu\text{g/ml}$ ), photograph taken on day 2; (c), colchicine ( $0.04 \mu\text{g/ml}$ ) and dexamethasone added together, photograph taken on day 2; (d), cytochalasin B ( $2 \mu\text{g/ml}$ ) and dexamethasone added together, photograph taken on day 3. Although a fair number of dead cells were usually present in the medium supernatant of the colchicine and cytochalasin B cultures, few of (visible as detached or loosely attached spheres) are visible in these photographs since the original media were removed before photography. Phase contrast,  $\times 150$ .

addition (figure 3c) the cells appear broader or larger with more cytoplasm than their dexamethasone counterparts and some possess the colchicine morphology in having multiple nuclei and spike-like projections. Other cells, however, still show a darkening or thickening at their centers reminiscent of dexamethasone treatment. The reduction in dexamethasone-induced morphological changes is more pronounced with cytochalasin B (figure 3d). Here the cells are even larger than with the combination of colchicine and dexamethasone and, while some have dark, thick centers, this darkening is less intensely concentrated than in the colchicine and dexamethasone case. Again, some cells which are very large with multiple nuclei seem to exhibit only the cytochalasin B morphology. Both colchicine and cytochalasin B reduce dexamethasone action in a dose dependent manner, since lower doses allow for a larger proportion of the culture to assume the dexamethasone morphology. It is interesting to consider that, while both agents are toxic for these cells within a few days, the presence of dexamethasone protects against this toxicity and greatly increases the survivability of the cultures. Unfortunately, no single unequivocal interpretation can be given for the above observations, so that although it is possible that dexamethasone acts in this system through microtubules and/or microfilaments, it is also reasonable to conclude that the colchicine and cytochalasin B effects are simply being superimposed upon those produced by dexamethasone.

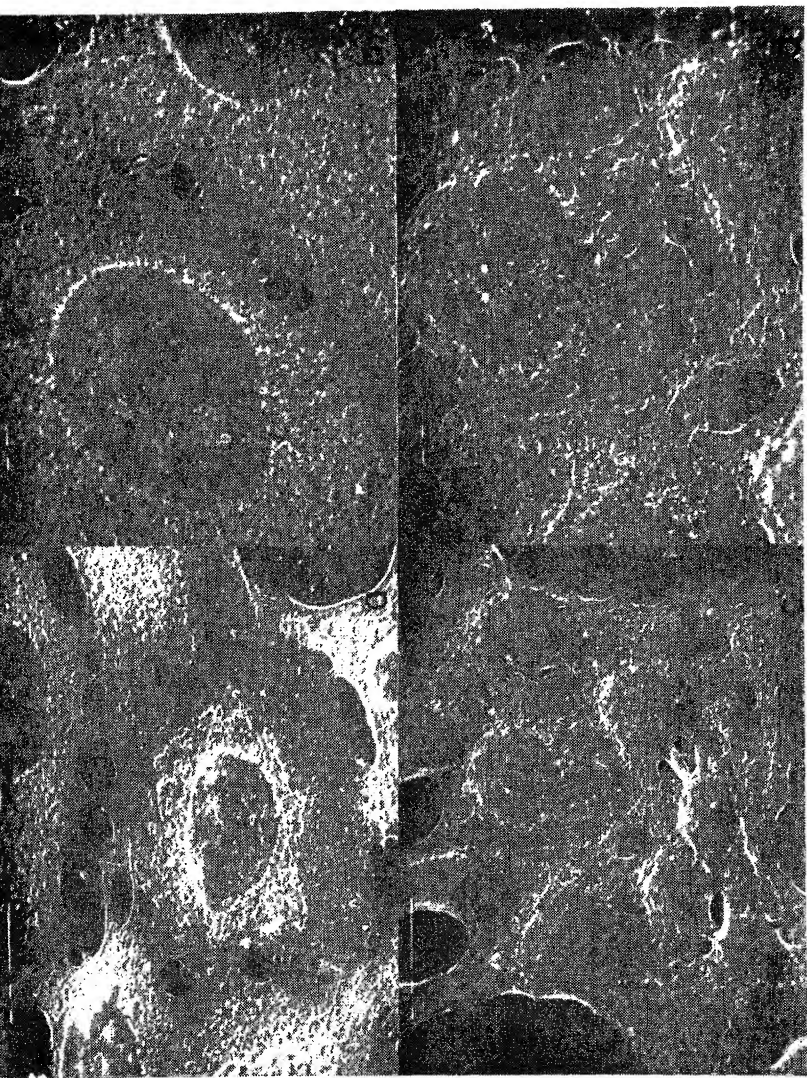
### *Structural changes seen with electron microscopy*

When scanning electron microscopy was employed, the initial observations made with the optical microscope were reinforced and, in addition, differences in surface structure were noted. In general, untreated specimens (figure 4a,b) display a flattened nuclear region devoid of microvilli or other surface specializations except for circular elevations which also appear in treated cells. Immediately peripheral to the nuclear zone is a broad band of microvilli and microplicae which extend from one-half to one-third of the distance from the limit of the nuclear zone to the border of the cell. This is surrounded by a relatively unremarkable peripheral zone.

Treated cells (figure 4c,d), on the other hand, are generally more rounded up than untreated with prominently raised nuclear regions. This is probably responsible for the darkening or 'thickening' seen under phase contrast. Even more striking changes are evident in the zone of microplicae and microvilli. This perinuclear zone in dexamethasone-treated cells is greatly reduced and the structural organization of these surface irregularities also appear to be altered. For instance, the fine 'halo' that surrounds the untreated nucleus is replaced by a coarser 'crown of thorns' which in the light microscope appear as 'lines' running over the cytoplasmic region.

Closer examination of the cell surface under higher magnification and using transmission electron microscopy revealed a major difference in the ability of the treated cell surface to interact with lead stains. As seen in figure 5 (a-c) black granules are clearly evident along the periphery of dexamethasone-treated cells stained with lead citrate. They do not appear in untreated cells (figure 5d,e) or if lead citrate is replaced with phosphotungstic acid (figure 5f). These grains are apparently lead precipitate and may have been caused by a glucocorticoid-induced change in the





**Figure 4.** Scanning electron microscopy of control and dexamethasone-treated cells. Photographs were taken on day 3 after dexamethasone addition. The details of the procedure are described in 'materials and methods'. All photographs shown here were taken with a 30°C tilt. The magnification bar code symbols are --- in which the first bar is 10  $\mu\text{m}$  and -- in which the second bar is 1  $\mu\text{m}$ . (a,b), Untreated cells at the following magnifications: (a)  $\times 1,500$ ; (b)  $\times 3,000$ ; (c,d), dexamethasone-treated cells at the following magnifications: (c)  $\times 1,500$  and (d)  $\times 3,000$ .



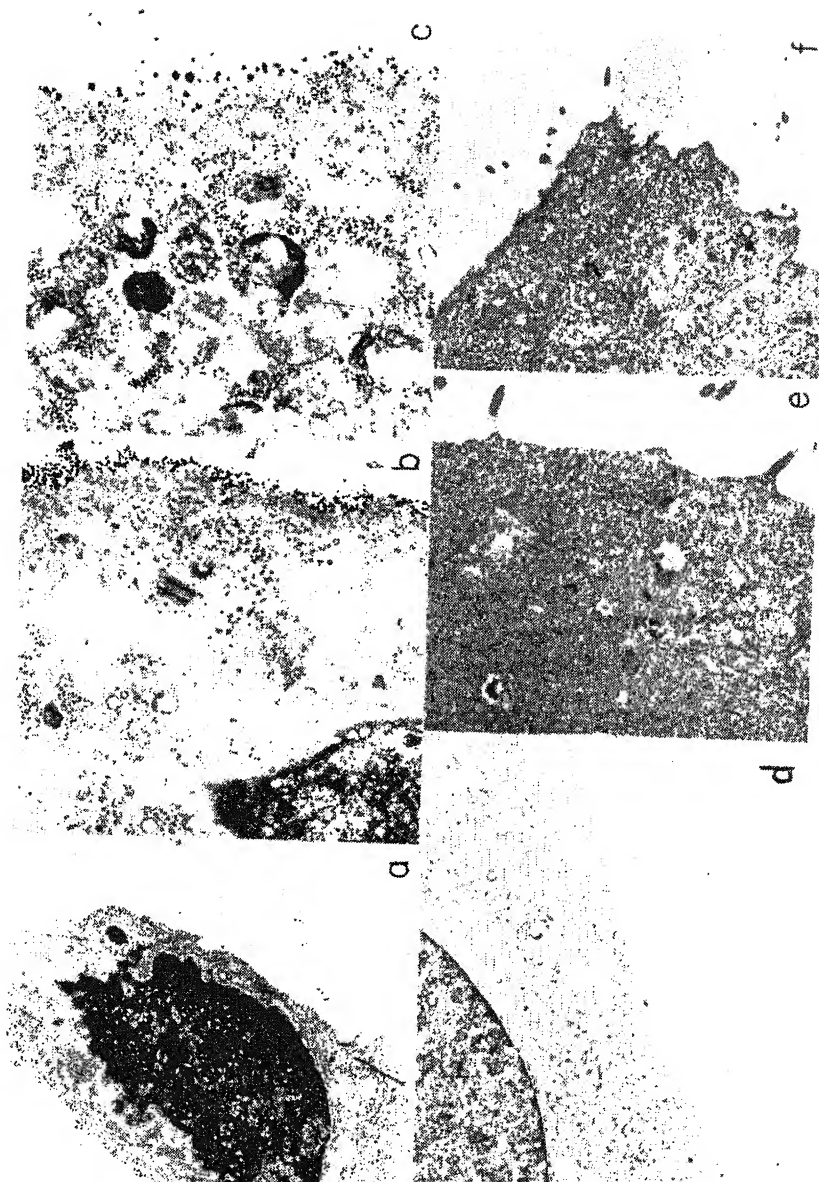


Figure 1. Electron micrographs of the cell surfaces of dexamethasone-treated cells as viewed with transmission electron microscopy. Cells were fixed and photographed taken using and dexamethasone addition. Staining procedures are outlined in 'materials and methods'. (a-c), Uranyl acetate-lead citrate stained cells at the following magnifications: (a),  $\times 10,233$ ; (b),  $\times 36,328$ ; (c),  $\times 77,760$ . (d-f), Untreated cells stained as in (a-c) at these magnifications: (d),  $\times 28,905$ ; (e),  $\times 77,760$ ; (f),  $\times 36,328$ .

The response shown by SV3T3 cells is clearly different. For instance, hepatoma cells (Freund *et al.*, 1975), SV40-transformed human skin fibroblasts (Furcht, 1979), and RLC-GAI epithelial cells (Berliner and Gerschenson, 1975) exhibit an increased resistance to trypsinization, a reduction in the number of microvilli, a tendency to form only monolayers and not pile up, or all 3 properties. These changes, some of which can be explained by an increase in fibronectin production or a rearrangement of its placement in the extracellular matrix (Furcht *et al.*, 1979), are also observed with SV3T3 cells. However, in all of the other cell types plus C6 glioma cells (Holbrook *et al.*, 1980), there is a pronounced flattening or spreading out of the cells which never occurs in dexamethasone-treated SV3T3 cultures. To the contrary, dexamethasone-treated SV3T3 cells appear more rounded with raised nuclear regions. Also, to our knowledge, no one has yet reported the nuclear darkening, the re-organization of the cell surface irregularities, and the precipitation of lead at the cell surface observed in the SV3T3 case.

The changes seen in SV3T3 cells may, in fact, be closely associated with the growth inhibition provoked by glucocorticoids. From biochemical studies (Das *et al.*, 1983), it appears that SV3T3 cells are blocked in late G<sub>2</sub> or early mitosis and overaccumulate DNA and protein. The darkening or thickening seen under phase contrast probably reflects this increase in macromolecular density. The transmission electron micrographs also show a high percentage of cells with condensed chromatin. Furthermore, it should be noted, the rounding up of treated cells is a likely consequence of this G<sub>2</sub> or mitosis block, since cells round up prior to cell division.

In regard to a possible mechanism whereby the steroid effects these structural changes and inhibits cellular proliferation, this morphological study provides no simple answers. Although colchicine and cytochalasin B do not produce the same structural changes as dexamethasone, a more specialized and selective involvement of glucocorticoids in microtubule and/or microfilament organization cannot be totally ruled out. Indeed, interference by dexamethasone in microtubule formation, especially during mitosis, still remains an attractive possibility. Another candidate might be a mechanism operating through the cell surface. The reduction in microvilli and the ability of the treated cell surface to interact with and precipitate lead indicate profound changes in the cell surface. However, whether this signals equally far-reaching functional ramifications in the membrane, such as hormone receptor or nutrient transport activity, must be tested by biochemical means. Thus, although this structurally-oriented investigation has not yielded a solution to the problem of how glucocorticosteroids modulate cellular growth, it has revealed major morphological changes elicited by a single hormone and provided clues for new avenues of experimentation.

## Acknowledgement

The authors thank Drs. John Aghajanian, Don Gantz and Elizabeth Beaumont for their skillful technical assistance in the operation of the transmission electron micro-

scope. This research project was supported by a grant CA30380 from the National Cancer Institute, DHHS, USA.

## References

- Baratz, R. S. (1976) *Stain Technol.*, **51**, 193.
- Berliner, J. A. and Gerschenson, L. E. (1975) *J. Cell. Physiol.*, **86**, 523.
- Das, H. R., Lavin, M., Sicuso, A. and Young, D. V. (1983) *J. Cell. Physiol.*, **117**, 241.
- Fodge, D. W. and Rubin, H. (1975) *Nature (London)*, **257**, 804.
- Freund, J. S., Dempsey, E. W., Loeb, J. N. and Borek, C. (1975) *Proc. Soc. Exp. Biol. Med.*, **150**, 14.
- Furcht, L. T., Moscher, D. F., Wendelschafer-Crabb, G. and Foidart, K. M. (1979) *Cancer Res.*, **39**, 2077.
- Grasso, R. J., Tedesco, T. A., Wodzinski, S. F., Johnson, C. E. (1978) *In Vitro*, **14**, 625.
- Hall, C. E., Jakus, M. A., Schmitt, F. O. (1945) *J. Appl. Phys.*, **16**, 459.
- Hayashi, I., Larner, J. and Sato, G. (1978) *In Vitro*, **14**, 23.
- Holbrook, N. J., Cox, W. I., Backney, J. F. and Grasso, R. J. (1980) *Proc. Soc. Exp. Biol. Med.*, **164**, 235.
- Holly, R. W., Kiernan, J. A. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 2908.
- Pratt, W. B., Aronow, L. (1966) *J. Biol. Chem.*, **241**, 5244.
- Reynolds, E. S. (1963) *J. Cell Biol.*, **17**, 208.
- Sarah, B. Eastman-Reks and Wayne V. Vedeckis (1986) *Cancer Res.*, **46**, 2457.
- Stempak, J. C. and Ward, R. T. (1964) *J. Cell Biol.*, **22**, 697.
- Young, D. V. and Dean, M. C. (1980) *J. Cell Physiol.*, **102**, 223.

C. S. RAMARAO, S. R. ACHARYA, K. S. KRISHNAN and  
U. W. KENKARE\*

Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road,  
Bombay 400 005, India

**Abstract.** Preparations having properties resembling those of synaptosomes have been isolated from whole fly homogenates of *Drosophila melanogaster* using ficoll gradient floatation technique. These have been characterized by marker enzymes and electron microscopy and binding of muscarinic antagonist  $^3\text{H}$  Quinuclidinyl benzilate. An uptake system for neurotransmitter,  $\gamma$ -Aminobutyric acid has been demonstrated in these preparations.

A high affinity uptake system for L-glutamate has also been studied in these subcellular fractions. This uptake of glutamate is transport into an osmotically sensitive compartment and not due to binding of glutamate to membrane components. The transport of glutamate has an obligatory requirements for either sodium or potassium ions. Kinetic experiments show that two transport systems, with  $K_m$  values  $0.33 \times 10^{-6}\text{M}$  and  $2.0 \times 10^{-6}\text{M}$ , respectively, function in the accumulation of glutamate. ATP stimulates lower affinity transport of glutamate. Inhibition of glutamate uptake by L-aspartate but not by phenylalanine and tyrosine indicates that a common carrier mediates the transport of both glutamate and aspartate.  $\beta$ -N-oxalyl-L- $\alpha$ ,  $\beta$ -diamino propionic acid and kainic acid, both inhibitors of glutamate transport in mammalian brain preparations, strongly inhibited transport of glutamate in *Drosophila* preparations.

Comparison with uptake of  $\gamma$ -aminobutyric acid and glutamate in isolated larval brain is presented to show that the synaptosome-like preparations we have isolated are rich in central nervous system derived structures, and presynaptic endings from neuromuscular junctions.

**Keywords.** *Drosophila melanogaster*;  $\gamma$ -aminobutyric acid; glutamate; neurotransmitter uptake.

## Introduction

Synaptosomes provide a useful system for the study of physiological processes underlying synaptic transmission (Wheeler, 1978). Since the first report of their characterization (Gray and Whittaker, 1962), they have been extensively employed to analyze diverse aspects of nerve cell structure and function. They have been used to study synaptic morphology (Whittaker, 1968; Kornbluth *et al.*, 1979) and synaptic membrane components (Yoshida and Imura, 1979; Gilbert and Wythe, 1979) as also metabolism of nervous tissue (Bradford, 1970; Booth and Clark, 1979) and neurotransmitter release (Whittaker *et al.*, 1972; Roberts *et al.*, 1979). Synaptosomal preparations have been employed with profit to identify uptake processes for

---

\*To whom all correspondence should be addressed.

Abbreviations used: AchE, Acetylcholinesterase; GABA,  $\gamma$ -aminobutyric acid; QNB, quinuclidinyl benzilate; ODAP,  $\beta$ -N-oxalyl-L- $\alpha$ ,  $\beta$ -diamino propionic acid; CS, Canton special; SDH, succinate dehydrogenase.

putative neurotransmitters (Wheeler, 1979) and to unravel mechanisms of action of certain neurotoxins (Tan and Abkowitz, 1979; Ramos *et al.*, 1979).

In a large majority of the above studies, synaptosomes have been isolated from mammalian nervous tissue. We have been attempting to use *Drosophila melanogaster* as a system to understand synaptic function. Behavioural genetic approaches have been very effective in identifying several loci in *Drosophila* genome which are likely to affect the function of the nervous system. Several of the behavioural mutants of *Drosophila* could have impaired synaptic transmission (Hall *et al.*, 1982). We have earlier been able to use a class of mutants to study the nature of the *Drosophila* acetylcholinesterase (AChE) (Zingde *et al.*, 1983). Neurochemical investigations *in vitro* of such mutants could lead to a clearer understanding of molecular events underlying synaptic function. It was therefore important to make synaptosomal preparations from *Drosophila* tissue. However, the conditions established for mammalian systems are not necessarily suitable for insect systems (Donnellan *et al.*, 1976; Breer and Jeserich, 1980). To our knowledge, so far there have been only two reports of isolation of synaptosomes from insect nervous system. Donnellan *et al.* (1976) used separated total heads of house flies to isolate synaptosomes on discontinuous ficoll density gradients. Using dissected head and thoracic ganglia of *Locusta migratoria*, Breer and Jeserich (1980) have developed a procedure for microscale isolation of synaptosomes on a ficoll gradient. These procedures *per se* could not be adapted for *Drosophila* as large scale separation of heads in *Drosophila* requires drastic treatment such as freezing in liquid nitrogen which may destroy fragile synaptosomal structures.

There is increasing evidence that glutamate may function as a major excitatory neurotransmitter in vertebrate central nervous system and insect neuromuscular junctions (Johnson, 1978; Jan and Jan, 1979; Davies and Watkins, 1979; Puil, 1981). Presence of selective high affinity uptake system for glutamate in the synaptosomes currently constitutes strong evidence for its neurotransmitter role in the mammalian brain (Logan and Snyder, 1971; Bennett *et al.*, 1973; Biziere *et al.*, 1980). There is overwhelming electro-physiological evidence for the neurotransmitter role of glutamate in the invertebrate neuromuscular junction (Takeuchi and Takeuchi, 1964; Taraskevich, 1975; Usherwood, 1972). Evidence has been presented for the neurotransmitter role of glutamate in *Drosophila* larval neuromuscular junction (Jan and Jan, 1976a,b). However, selective high affinity uptake system for glutamate has not so far been reported in insect nervous system.

We have attempted to isolate synaptosome like structures from *D. melanogaster* whole flies using ficoll floatation technique which is an adaptation of the method of Breer and Jeserich (1980). In this paper, we describe the characterization and properties of such a preparation from *Drosophila*. We show that this preparation is associated with an uptake system for  $\gamma$ -aminobutyric acid (GABA), which is well established as an inhibitory neurotransmitter in the insect central nervous system (Pichon, 1974) and describe the properties of a sodium dependant, ATP stimulated high-affinity uptake system for glutamate.

Preliminary results of uptake studies with larval brains also show fast/high affinity uptake for glutamate and GABA.

U-[ $^{14}\text{C}$ ]-GABA (224 mCi/mmol), [ $^3\text{H}$ ]-glutamic acid (43 Ci/mmol) and [ $^3\text{H}$ ]-quinuclidinyl benzilate (QNB) (44 Ci/mmol) were obtained from Amersham, UK. U-[ $^{14}\text{C}$ ]-glutamic acid (130 mCi/mmol) was purchased from Bhabha Atomic Research Centre, Bombay. Ficoll and kainic acid were obtained from Sigma Chemical Company, St. Louis, Missouri, USA.  $\beta$ -N-oxalyl-L- $\alpha$ ,  $\beta$ -diamino propionic acid (ODAP) was a gift from Prof. G. Padmanaban of Indian Institute of Science, Bangalore. All other chemicals were of the best analytical grade available.

### *Preparation of synaptosomal fractions*

The canton special (CS) strain of *D. melanogaster* was used in all experiments. Flies were collected within two days of emergence and immediately chilled in ice. All operations were carried out in cold. The flies were homogenized in ice-cold 20 mM Tris buffer, pH 7.4 containing 0.32 M sucrose (extraction buffer), in a Braun homogenizer at a speed of 1000 rpm, using 6 strokes. The homogenate was filtered through a nylon net and centrifuged at 1100 *g* for 10 min in a sorvall RC5 centrifuge using SM24 rotor to remove the nuclear debris. The supernatant was gently layered over a cushion of 46% sucrose and centrifuged at 16,000 *g* for 30 min in a HB4 swing-out rotor to obtain the mitochondrial pellet. The band above 46% sucrose which we call mitochondrial fraction was collected with a syringe and gently but thoroughly mixed with an equal volume of 24% ficoll (w/v) in 0.32 M sucrose Tris pH 7.4 and centrifuged thereafter for 90 min at 10,000 *g*. In addition to a pellet at the bottom of the tube, a pellicle was formed at the top of the gradient. The pellicle was collected with a syringe and centrifuged in 10 volumes of extraction buffer at 12,500 *g* for 20 min. The pellet thus obtained is relatively enriched in synaptosome like structures and is made into a suspension using 0.5 ml of incubation buffer (see below) per g of original tissue. This suspension (pellicle fraction) was used for further studies.

### *Uptake studies*

The uptake of [ $^{14}\text{C}$ ]-labelled GABA was measured by incubating the pellicle fraction in a modified Krebs-Henseleit solution (incubation buffer). All incubations were carried out at 35°C. The modified Krebs-Henseleit solution consisted of 127.2 mM NaCl, 5.0 mM KCl, 2.7 mM  $\text{CaCl}_2$ , 1.3 mM  $\text{MgSO}_4$ , 25 mM Tris-chloride (pH 7.4) and 11.1 mM glucose (Wheeler, 1978). The final concentration of GABA used was  $5 \times 10^{-6}$  M inclusive of radioactive GABA (0.25  $\mu\text{Ci/ml}$ ) and that of glutamate was  $1 \times 10^{-6}$  M (0.125  $\mu\text{Ci/ml}$  reaction mixture). Incubation was started by adding 200  $\mu\text{l}$  of pellicle fraction to 800  $\mu\text{l}$  of incubation medium. At appropriate time intervals, 200  $\mu\text{l}$  of the reaction mixture was withdrawn and filtered through Schleiser and Schull BA85 filters with a pore size of 0.45  $\mu\text{m}$ , using a Hoeffler filtration manifold. The filters were washed twice with 5 ml of the incubation medium without radioactivity, left at room temperature, dried under an infra-red lamp and counted in a liquid scintillation counter after addition of 8 ml of toluene scintillation fluid. For

uptake studies with larval brains, 3rd instar larvae from a synchronous population were taken and the brains dissected out in the modified Krebs'-ringer solution and 5 brains were used for each sample. Final concentration of GABA used was  $\sim 5 \mu\text{M}$  and of glutamate  $\sim 50 \text{ nM}$ . Incubations were done at room temperature ( $\sim 26^\circ\text{C}$ ) for upto 50 min. The brains were collected after incubation by filtering through glass fibre filter paper ( $0.45 \mu$ ), washed and counted as done for synaptosomes. [ $^3\text{H}$ ]-QNB binding by various fractions of *Drosophila* preparations was assayed by incubating  $50 \mu\text{l}$  sample with  $\text{H}^3$ -QNB at a final concentration of  $\sim 3.8 \text{ nM}$ . Filtration assay was carried out as usual.

### Electron microscopy

The sample was fixed in ice-cold 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) for 2 h. The fixed material was sedimented by centrifugation, post fixed in 1% osmium tetroxide for 2 h in the same buffer, dehydrated in graded ethanol and propylene oxide and finally embedded in Araldite. Ultra thin sections (60–90 nm) were stained with uranyl acetate and then lead citrate prior to examination in a Jeol electron microscope.

### Enzyme assays

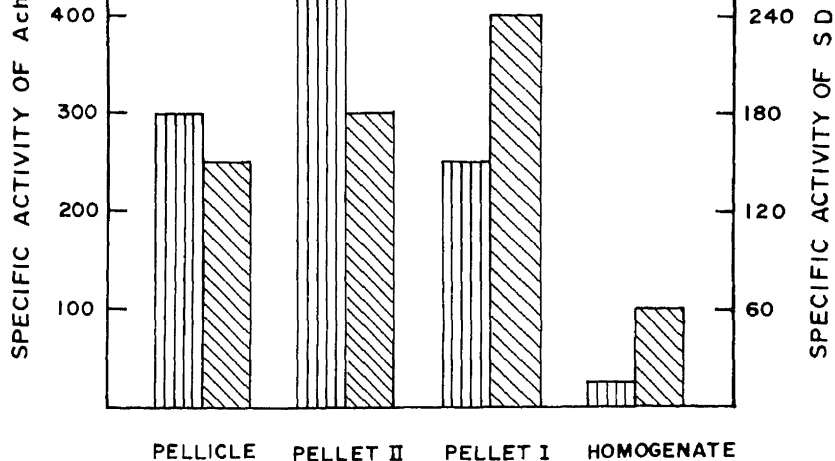
AchE (EC 3.1.1.17) was estimated by the procedure of Ellman *et al.* (1961). Succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed according to procedure of King (1967). Protein was estimated by Hartree's method (1972).

## Results

The fractionation procedure carried out using 12% ficoll resulted in 3 distinct fractions. The distribution of marker enzymes in each of the fractions is presented in figure 1. It would appear that pellet I, pellet II and pellicle correspond to mitochondria, broken membrane fragments and sealed membrane structures respectively. Pellet I has maximal activity of SDH and relatively less activity of AchE, while reverse is the case for pellet II fraction. Both the enzymes are enriched in the pellicle fraction. Table 1 shows binding of QNB to various fractions indicated, and shows that the synaptosome like fraction (pellicle fraction) has a substantial enrichment of binding in relation to the homogenate.

Many cholinergic macromolecules are found exclusively in the central nervous system of *Drosophila*. It is hence likely that muscarinic receptor seen by QNB binding is also a marker for CNS derived structures. Our results presented in table 1 and the presence of uptake system for GABA and glutamate in larval brains (table 4), suggests that a substantial fraction of our *in vitro* preparation contain endings from the central nervous system of *Drosophila*.

Ultrastructurally, the pellicle fraction contained different populations of membrane bound structures. Large featureless membrane sacs were seen as well as



**Figure 1.** Enzyme activity profiles of final fractions. Vertical striped AChE and cross hatched, SDH. Activities are expressed in units of  $\mu\text{mol}$  acetyl thiocholine hydrolysed/g/min or  $\mu\text{mol}$  succinate oxidised/g/min.

The pellicle activities were measured in pellicles washed in isotonic buffer and resuspended in homogenising buffer. Pellet I is the pellet at the bottom of the ficoll gradient. Pellet II is the homogeneously distributed particulate material in the ficoll gradient. After recovering the pellicle and pellet I fractions, the body of ficoll gradient was diluted 20 fold by isotonic buffer and spun at 20,000  $g$  for 45 min and a pellet of membranous material that was obtained is called pellet II.

**Table 1.** Typical profile of QNB binding to different fractions of the *Drosophila* preparation.

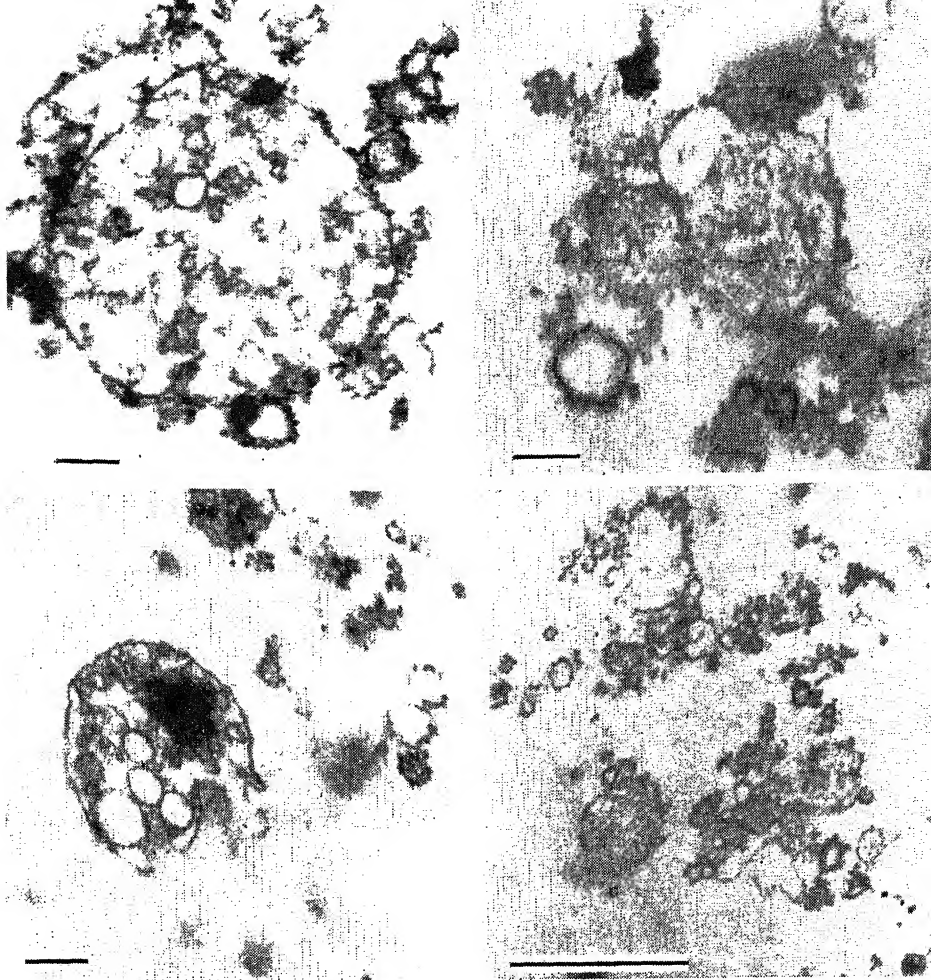
Fraction	QNB-binding in fmol/mg protein
Homogenate	9.7
Post-3000 rpm supernatant	19.1
Synaptosome-like fraction (pellicle fraction)	36.2

containing a substantial number of nerve endings were collected in the pellicle. This was to be expected as whole flies were used for fractionation.

In view of the fact that GABA is well recognised as an inhibitory neurotransmitter in the insect central nervous system we looked for GABAergic uptake by the pellicle fraction which appeared to be relatively enriched in synaptosomes. When the pellicle fraction was incubated with  $[^{14}\text{C}]$ -GABA for various time intervals, there was a significant uptake of GABA which was linear for about twenty minutes (figure 3). The linear dependence of this uptake on protein concentration is shown in figure 4.

Table 2 shows that GABA uptake is dependent upon the simultaneous presence of external sodium and chloride ions. Replacement of chloride by acetate ions and

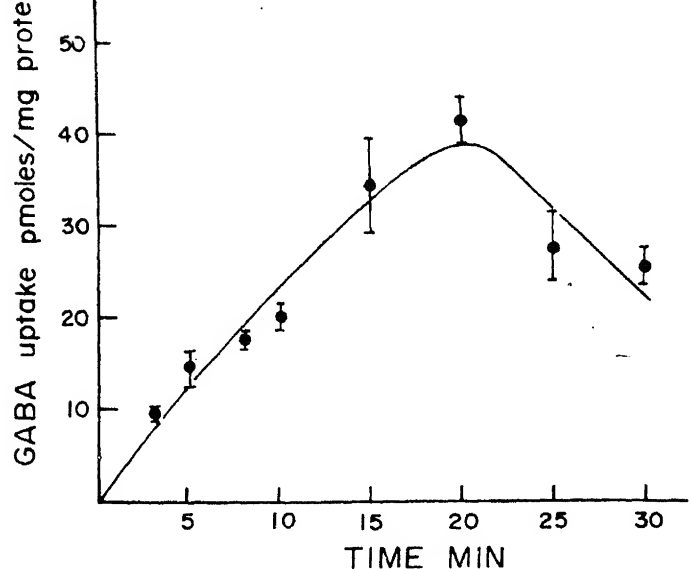




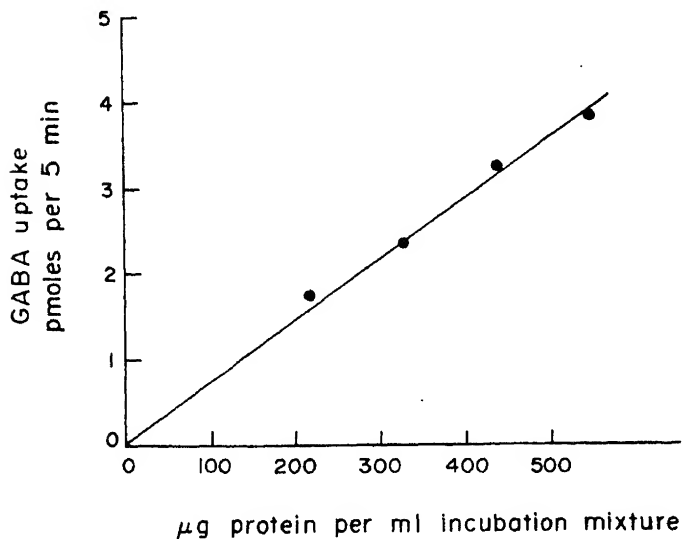
**Figure 2.** Montage of electron micrographs of the pellicle fraction obtained by floatation over 12% ficoll. Bars represent 0.25  $\mu\text{m}$ .

replacement of sodium by Tris ions resulted in considerable decrease in the uptake of GABA. It may however be noted that in these experiments 25 mM chloride was already present as part of the incubation buffer and this may be one of the reasons why replacement of chloride by acetate ions did not result in greater decrease of GABA uptake.

The data presented in figure 5 illustrate the time course of [ $^{14}\text{C}$ ]-glutamate uptake by a subcellular fraction (pellicle fraction) isolated from whole flies of *D. melanogaster*. The uptake was linear with time usually upto 8–10 min after which there was a sharp decline. All uptake experiments were therefore carried out for times of 8 min or less to avoid the declining phase of the uptake. To ascertain the proportion of radioactivity retained by non-specific absorption on filter papers and binding



**Figure 3.** Time course of GABA uptake. GABA at a concentration of  $5 \times 10^{-6}$  M inclusive of [ $^3$ H]-GABA 0.25  $\mu$ Ci/ml was used. Incubations were started by the addition of 200  $\mu$ l of synaptosomal preparation to 800  $\mu$ l of the incubation medium. Aliquots of 200  $\mu$ l were withdrawn at appropriate intervals, filtered and counted.

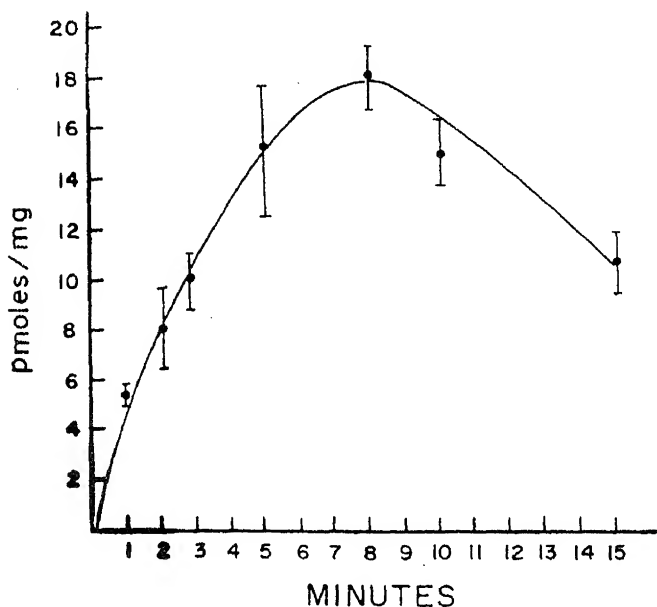


**Figure 4.** Dependence of GABA uptake on protein concentration. Uptake is calculated from the differences between counts retained after 0 min and 5 min incubations. GABA at a final concentration of  $5 \times 10^{-6}$  M and 0.25  $\mu$ Ci/ml was used in the incubation.

**Table 2.** Dependence of GABA uptake on sodium and chloride ions.

Incubation medium	GABA uptake (pmol/mg protein) for 8 min		
	Experiment 1	Experiment 2	Experiment 3
Standard incubation medium containing 127.2 mM sodium chloride	6.90	7.40	8.33
Standard incubation medium in which NaCl replaced by sodium acetate	3.51	4.16	3.79
Standard incubation medium in which NaCl replaced by Tris-Cl	5.18	5.74	5.64

Incubation to study GABA uptake were carried out as described in 'experimental procedures'. 1.2 mg of pellicle protein was incubated with  $5 \times 10^{-6}$  M GABA. The uptake is calculated from the difference in cpm retained in filter papers of samples at 8 min and 0 min.



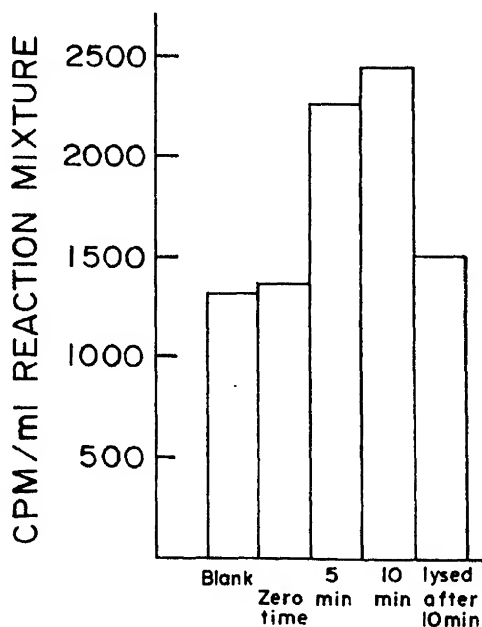
**Figure 5.** Time course of [ $^{14}\text{C}$ ]-L-glutamic acid uptake by *Drosophila* pellicle fraction. The incubation mixture contained 1 mg protein of the preparation,  $10^{-6}$  M L-glutamic acid inclusive of [ $^{14}\text{C}$ ]-glutamic acid ( $0.125 \mu\text{Ci}$ ) in one ml of Krebs-Henseleit solution [127.2 mM NaCl, 5.0 mM KCl, 2.7 mM  $\text{CaCl}_2$ , 1.3 mM  $\text{MgSO}_4$ , 25 mM Tris-chloride buffer (pH 7.4) and 11.1 mM glucose]. Incubations were started by the addition of the

blanks where no protein was added.

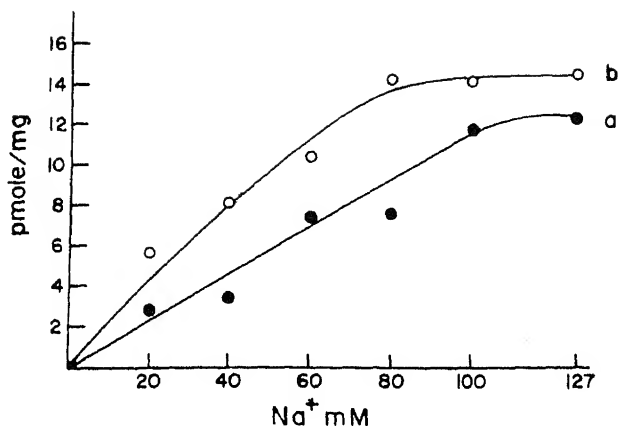
Figure 7 shows the dependence of glutamate on  $\text{Na}^+$  concentration. Two curves, showing glutamate uptake for 3 min (figure 7a) and 5 min (figure 7b) respectively are shown. It is clear that uptake of glutamate in this fraction is absolutely dependent on  $\text{Na}^+$  concentration and that sucrose cannot replace  $\text{Na}^+$ . In another experiment  $\text{Na}^+$  concentration was varied and osmolarity difference made up either with sucrose (figure 8a) or with Tris-chloride (figure 8b). It is seen from figure 8 that maximum uptake of glutamate is obtained when the  $\text{Na}^+$  concentration is around 100 mM. Experiment described in figure 8 also showed that chloride had no effect on glutamate uptake as Tris-chloride was ineffective. Sodium acetate and potassium chloride were also as effective as sodium chloride in stimulating the uptake.

Figure 9 shows the dependence of glutamate uptake on protein concentration in the incubation mixture under the conditions of our experiments. The uptake of glutamate was maximal at a protein concentration of 0.8 mg/ml. However the usual concentration of protein employed in other experiments was of the order of 0.3–0.4 mg/ml.

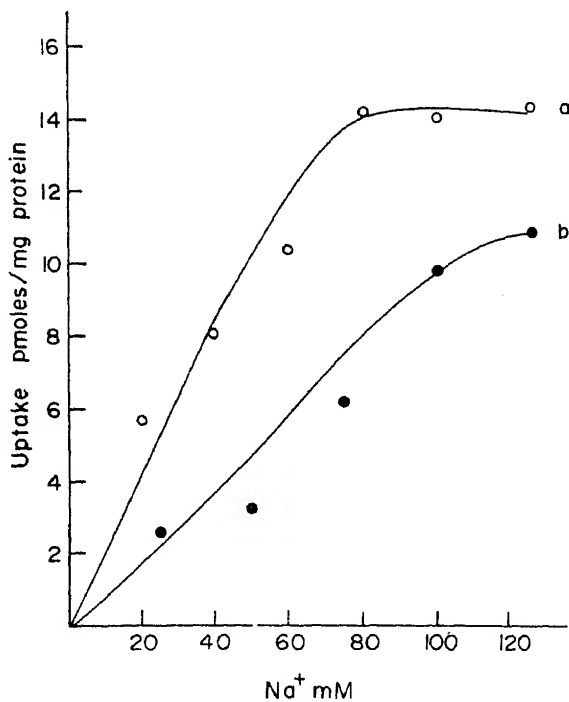
Figure 10a shows the effect of glutamate concentration on its uptake by the pellicle fraction. Two uptake systems are evident. One with high affinity has a  $K_m$  of



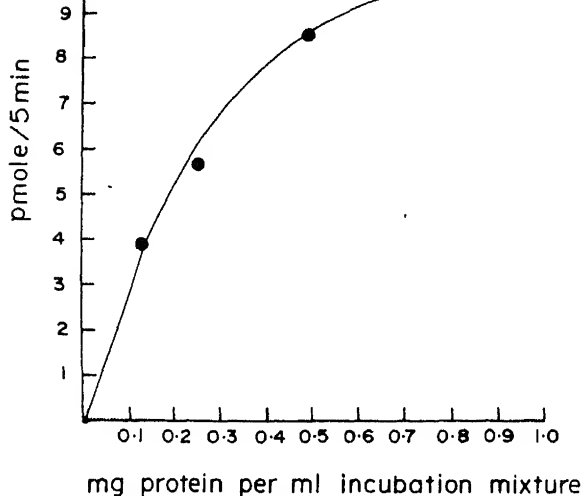
**Figure 6.** Retention of radioactivity (glutamate) on filter papers. Comparison of blank, zero time and lysed samples. Lysed samples are 10 min samples to which a 20 fold volume of ice cold water was added and after a short vigorous shaking the samples were filtered. In some cases lysis was done by washing filtered pellicle fraction with ice cold water in place of isotonic buffer.



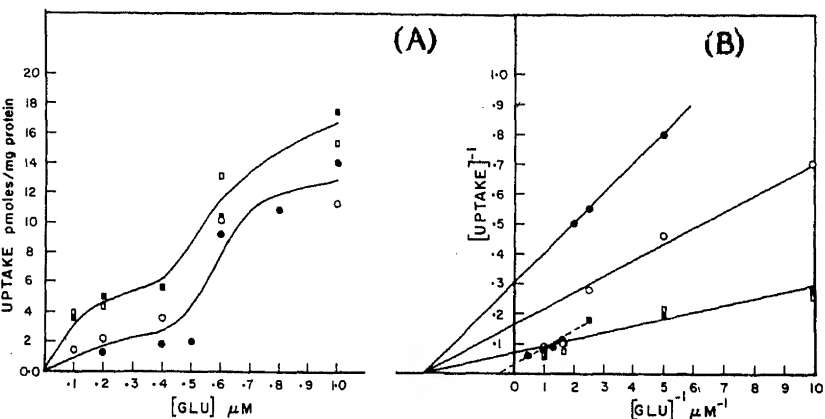
**Figure 7.** Uptake in the presence of increasing concentration of NaCl. In the Krebs-Henseleit solution, NaCl was replaced by equiosmolar sucrose to obtain lower NaCl concentrations. (a), uptake for 3 min (b), uptake for 5 min. Zero time blanks were subtracted in each case.



**Figure 8.** Uptake in the presence of increasing concentration of NaCl. Uptake is expressed as p mol/mg protein/5 min. Zero time blanks were corrected for in each case. (a), NaCl



**Figure 9.** Dependence of [ $^{14}\text{C}$ ]-L-glutamic acid uptake on protein concentration. [ $^{14}\text{C}$ ]-glutamic acid at  $10^{-6}\text{M}$  ( $0.125\text{ }\mu\text{Ci/ml}$ ) was incubated with varying amounts of the pellicle fraction. The uptake for 5 min was calculated from the difference in cpm between zero time and 5 min samples for each protein concentration.



**Figure 10.** **A.** Dependence of uptake on glutamate concentration. L-Glutamate concentration varied from  $10^{-7}\text{M}$  to  $1 \times 10^{-6}\text{M}$  in the Krebs-Henseleit medium. The radioactivity was also varied from  $0.0125\text{ }\mu\text{Ci/ml}$  to  $0.125\text{ }\mu\text{Ci/ml}$ . When higher concentrations of glutamate were used, the radioactivity was retained at  $0.125\text{ }\mu\text{Ci/ml}$ . Circles represent uptake for 3 min and squares represent uptake for 5 min. The values are for different preparations. **B.** Double reciprocal plot of the uptake data. The two foci correspond to  $K_m$ :  $0.33\text{ }\mu\text{M}$  and  $2\text{ }\mu\text{M}$ .

different from the one that takes up GABA, glycine and other amino acids (Balcar and Johnston, 1972). In such a case high concentrations of aspartate should inhibit the uptake of glutamate. To study the specificity of the glutamate uptake system in *Drosophila* preparations, phenylalanine, tryptophan and aspartic acid were added to the incubation mixture. Figure 11 shows that while phenylalanine and tryptophan did not have any effect, aspartate even at  $10^{-5}$  M concentration inhibited the glutamate uptake completely. This indicates that glutamate and aspartate are transported by the same system as is the case with mammals (Fonnum *et al.*, 1981).

ODAP is a neurotoxin isolated from the seeds of *Lathyrus sativus* which causes neurolathyrism. ODAP was shown to be a potent inhibitor of high affinity glutamate uptake system in rat brain synaptosomes (Lakshmanan and Padmanabhan, 1974). Figure 12a shows the effect of ODAP on glutamate uptake by *Drosophila* pellicle

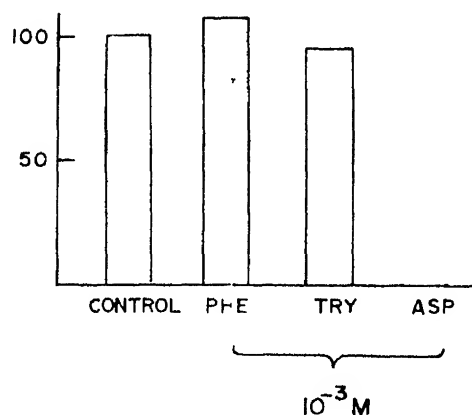


Figure 11. Inhibition of uptake by amino acids. Phenylalanine, tyrosine and aspartate were present in the respective samples at a concentration of  $10^{-3}$  M in the Krebs-Henseleit solution. Aspartate concentration  $10^{-5}$  M,  $10^{-4}$  M and  $10^{-3}$  M show a similar profile. Glutamic acid was  $10^{-6}$  M (0.125  $\mu$ Ci/ml).

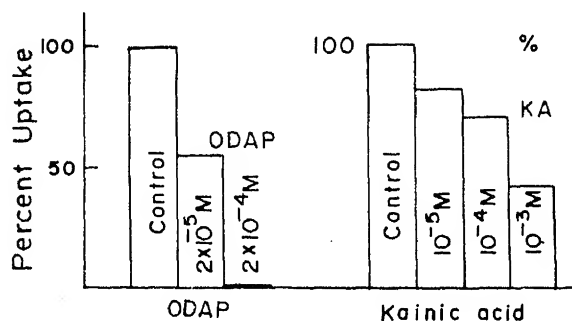


Figure 12. Effect of kainic acid and ODAP on uptake of L-glutamic acid by *Drosophila* pellicle fraction.

Table 3 shows the effect of sodium and ATP on glutamate uptake by the pellicle fraction from *Drosophila*. ATP stimulated the uptake of glutamate in the presence of  $\text{Na}^+$  nearly two fold. The uptake of glutamate in the absence of  $\text{Na}^+$  could also be stimulated by ATP. Thus ATP is able to at least partly restore glutamate uptake in the absence of sodium ions.

Table 4 shows results of GABA and glutamate uptake studies with isolated larval

**Table 3.** Role of ATP and sodium in L-glutamate uptake.

	Uptake (p moles/mg protein) in		
	3 min	5 min	8 min
Control + Na - ATP	2.79	4.80	5.86
a* + Na + ATP 2 mM	5.30	9.40	21.70
- Na + ATP 2 mM	2.06	3.26	4.93
- Na - ATP	Nil	Nil	Nil
Uptake (p moles/mg protein/5 min)			
0.2 $\mu\text{M}$ Glu		2.41	
b* 0.2 $\mu\text{M}$ Glu + ATP		2.37	
1.0 $\mu\text{M}$ Glu		3.68	
1.0 $\mu\text{M}$ Glu + ATP		15.59	
	Uptake (p moles/mg protein) in		
	3 min	5 min	8 min
c* Control	3.07	5.28	5.86
+ ATP	6.40	7.88	16.30

Uptake studies were performed in Krebs-Henseleit medium and corrected for zero time values.

+ The concentration of radioactivity was maintained at 0.025  $\mu\text{Ci/ml}$  at 0.2  $\mu\text{M}$  Glu and 0.125  $\mu\text{Ci/ml}$  at 1.0  $\mu\text{M}$  Glu.

\*Glutamic acid concentrations were  $10^{-6}\text{M}$  (0.125  $\mu\text{Ci/ml}$ ) in Krebs-Henseleit medium. NaCl was replaced by equiosmolar sucrose for studies in the absence of sodium.

**Table 4.** Typical set of GABA and glutamate uptake studies in isolated larval brains.

Time (min)	Uptake in fmoles/brain	
	GABA	Glutamate
0	230	1.07
40	1010	9.38
40, (0°C)	440	2.35
40, 2 mM aspartate*	—	4.35
40, 1 mM picrotoxin*	1180	—

GABA final concentration was  $\sim 5\text{ }\mu\text{M}$ .

Glutamate final concentration was  $\sim 50\text{ nM}$ .

\*Final concentration in the assay mixture.



## Discussion

Our early attempts to prepare synaptosomes from separated heads were not successful. The reason could be that nerve endings being fragile get disrupted and synaptosomes are not formed at all after the drastic treatment of freezing the flies in liquid nitrogen prior to separation of heads.

The procedure described above, using whole flies, yields intact, sealed membrane structures a substantial number of which are similar to synaptosomes. As with Brecht and Jeserich (1980) this could be attributed to mild homogenization procedures involving less damaging liquid shear forces. Maintenance of cold conditions throughout and using young flies appear to be very critical in obtaining the synaptosome like structures in pellicle. However, in spite of all these precautions, the success rate is only about 75–80% and the preparations vary considerably in qualitative and quantitative recovery of these structures.

Regarding the experiments on GABA transport described here, it is necessary to emphasize that we are dealing with GABA translocated into osmotically sensitive structures and not with GABA bound to membranes. Thus when the pellicle fraction incubated with GABA was osmotically shocked, filtered and counted, the counts retained were equal to those in the blank, proving that the radioactivity was transported into osmotically sensitive compartments.

The GABA transport system in *D. melanogaster* is found to be dependent on both  $\text{Na}^+$  and  $\text{Cl}^-$  ions. This has also been observed with synaptosomal preparations from mammalian tissue (Kanner, 1978) and synaptosomes and membrane vesicles from locust ganglia (Gordon *et al.*, 1982). GABA uptake is found to be linear up to 20 min.

The experiments indicate the presence of a high affinity glutamate uptake system in the pellicle fraction from *D. melanogaster*. This seems to need monovalent cations and is stimulated by ATP. Whereas glutamate uptake declines rapidly; GABA uptake is linear to about 15 min which probably indicates that the structures which contribute to the two uptake systems are different. This is also indicated by the relatively poorer levels of glutamate uptake in isolated brains compared to *in vitro* fractions while GABA uptake levels are similar in both systems. It may be noted that glutamate is known to be a neuromuscular junction transmitter in *Drosophila* (Jan and Jan, 1976b). Isolated brains are unlikely to carry these synapses with them, whereas the synaptosome-like fraction from whole flies may contain these structures.

Because of the fragility of synaptosomal structures and variation in absolute uptake rates specially with respect to glutamate, it is important that comparative studies are carried out with the same preparation. For example figure 3 is average of studies where the absolute optimum uptake was somewhat similar; whereas table 1 shows another set of results which are close. However the trends in the results have always been the same. In spite of some of these problems, the procedure presented in this paper led to viable preparations which could be used to study some of the properties associated with the synaptic system of *Drosophila*.

One intriguing feature of glutamate uptake is that it is not linearly dependent on

due to further metabolism of glutamate at glutamate preparations leading to loss of radio-label. This suggestion is supported by our preliminary results (not shown) that  $\alpha$ -ketoglutarate and GABA are the products formed when glutamate is taken up by these preparations.

In our experiments with glutamate uptake, background values where no pellicle fraction is added are substantially high (see figure 6) as compared to background values in case of GABA uptake which are quite low. Several attempts were made to reduce the background by increasing the number of washes and by presoaking the filter papers in cold glutamate but to no effect. However, all the conclusions have been derived from experiments in which glutamate uptake was measured at various time intervals and zero time values subtracted. Also, counts taken up by the pellicle fraction could be released by lysis of the preparation. We therefore consider our glutamate uptake experiments reliable and conclusions drawn therefrom meaningful.

On the analogy of sodium dependent glutamate uptake in other systems (Kenner and Sharon, 1978), it is possible that in the system under investigation, glutamate is cotransported by the carrier along with the sodium ions, the downhill transport of which energizes the uphill transport of glutamate (Krnjević, 1974). This suggestion is strengthened by the fact that ATP can replace sodium ions in supporting transport of glutamate (table 3).

The requirement for sodium ions does not appear to be specific, and can also be met by potassium ions. It is quite likely that the closed structures in our preparations are low in concentration of monovalent cations. Hence either sodium or potassium ions are likely to move inwards cotransporting glutamate along with them. We are not certain about the conditions of membrane potential in this system and hence do not know what its contribution to glutamate transport would be.

The requirement for ATP in the glutamate transport system of our preparations appears to be complex. The dependence of glutamate uptake on its concentration, itself is biphasic (figure 10). The uptake observed at higher concentrations of glutamate alone is stimulated by ATP (table 3). Also the stimulation by ATP at a given glutamate concentration becomes obvious only at longer time intervals. All these observations indicate that two transport systems for glutamate are operating in our preparation. One surmise is that there is a vesicular transport system for glutamate functioning inside the structures. It is known that vesicular transport of catecholamines requires ATP transport (Apps, 1982). The stimulation by ATP of glutamate transport observed at longer times of incubation supports this idea since glutamate and/or ATP concentration has to build up inside the sealed membrane structures for vesicular transport to take over. It is also possible that the biphasic dependence of uptake on glutamate concentration is a specific stimulation of lower affinity uptake by endogenous ATP.

Some of the intriguing features of glutamate transport described here such as biphasic dependence on glutamate concentration and complex nature of ATP requirement could be ascribed to contamination by glial cells which are known to transport glutamate in other systems (Fonnum, 1978). At the present stage of our investigations it is not possible to rule out this possibility. However two observations argue against substantial glial cell contamination. One is the dose dependent inhibition by kainic acid which is known not to inhibit glutamate transport by glioma cells

incubation of our preparations with glutamate produced GABA and  $\alpha$ -ketoglutarate but not glutamine. Since glutamine synthetase is mainly localized in glial cells (van der Berg *et al.*, 1975), the lack of detectable amounts of glutamine in the present case indicates the absence of glial cells in our preparations.

The preliminary results from larval brain uptake of glutamate and GABA indicate that this could also be a useful method of measuring neurotransmitter uptake. Lack of any effect of picrotoxin, a known GABA receptor antagonist, on GABA uptake in larval brains ruled out receptor binding as the cause for increase in radioactivity with incubation time. Inhibition of glutamate uptake by aspartate is in line with the known effect of aspartate on glutamate transport system (Fonnum *et al.*, 1980). Difficulties in the use of this system, however arise in replacing incubation media under conditions conveniently. We also notice that the synaptosome-like fraction made from whole flies may have a larger population of glutaminergic nerve endings compared to isolated brain. Isolated brain in the process of picking up may lose most of the synaptic endings at the neuromuscular junction. Hence the *in vitro* preparations described in this paper could still be more useful in such studies.

## Acknowledgement

We thank Dr. R. N. Singh for help in electron microscopy and Dr. G. Kudva and Dr. S. Swaminathan, summer visitors, for help in some experiments.

## References

- Apps, D. K. (1982) *Trends Biochem. Sci.*, **7**, 153.  
 Balcar, V. J. and Johnston, G. A. R. (1972) *J. Neurochem.*, **19**, 2657.  
 Bennet, J. P., Logan, W. J. and Snyder, S. H. (1973) *J. Neurochem.*, **31**, 513.  
 Biziere, K., Thompson, H. and Coyle, J. T. (1980) *Brain Res.*, **183**, 421.  
 Booth, R. and Clark, J. (1979) *FEBS Lett.*, **107**, 387.  
 Bradford, H. F. (1970) *Brain Res.*, **19**, 239.  
 Breer, H., Jeserich, G. (1980) *Insect Biochem.*, **10**, 457.  
 Davies, J. and Watkins, J. C. (1979) *J. Physiol.*, **297**, 621.  
 Disbros, J. K., Gershten, M. J. and Ruth, J. A. (1982) *Biochem. Biophys. Res. Commun.*, **108**, 1221.  
 Donellan, J. F., Alexander, K. and Chendlik, R. (1976) *Insect Biochem.*, **6**, 419.  
 Ellman, G. L., Courtney, K. D., Andres Jr. V. and Featherstone, R. M. (1961) *Biochem. Pharmacol.*, **7**, 88.  
 Fonnum, F. Ed. (1978) *Amino acids as chemical transmitters* (New York: Plenum Press).  
 Fonnum, F., Sorensen, D. M., Kvale, I., Soreide, A., Skrede, K. K. and Walaas, I. (1981) in *Regulatory mechanisms of synaptic transmission* (eds R. Tapia and W. Cotman) (New York: Plenum Press) p. 5.  
 Gilbert, J. and Wythe, M. (1979) *Br. J. Pharmacol.*, **67**, 479.  
 Gordon, G., Zlotkin, E. and Kanner, B. (1982) *Biochim. Biophys. Acta.*, **688**, 229-236.  
 Gray, E. G. and Whittaker, V. P. (1962) *J. Anat. London*, **96**, 79.  
 Hall, J. C., Greenspan, R. J. and Harris, W. A. (1982) *Genetic Neurobiology* (Cambridge, Massachusetts: MIT Press).  
 Hartree, E. F. (1972) *Anal. Biochem.*, **48**, 422.  
 Jan, L. Y. and Jan, Y. N. (1976a) *J. Physiol.*, **262**, 189.  
 Jan, L. Y. and Jan, Y. N. (1976b) *J. Physiol.*, **262**, 215.  
 Jan, Y. N. and Jan, L. Y. (1979) in *Insect neurobiology and pesticide action* (ed. F. E. Rickett) (London: The Society of Chemical Industry) p. 161.  
 Johnson, J. L. (1978) *Prog. Neurobiol.*, **10**, 155.  
 Kanner, B. I. and Sharon, I. (1978) *Biochemistry*, **17**, 3949.

- Korngluth, S., Scott, G. and Jaramillo, R. (1979) *J. Neurochem.*, **33**, 875.
- Krnjevic, K. (1974) *Physiol. Rev.*, **54**, 418.
- Lakshmanan, J. and Padmanaban, G. (1974) *Biochem. Biophys. Res. Commun.*, **58**, 690.
- Logan, W. J. and Snyder, S. H. (1971) *Nature (London)*, **234**, 297.
- Nicklas, W. J. (1981) *Neuroscience Res. Prog. Bull.*, **19**, 372.
- Pichon, Y. (1974) *Physiol. Insecta*, **4**, 102.
- Puil, E. (1981) *Brain Res. Rev.*, **3**, 229.
- Ramos, S., Grollman, E. F., Kazo, P. S., Dyer, S. A., Habig, W. H., Hardegree, M. C., Kabak, H. R. and Kohn, L. D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4783.
- Roberts, F., Hill, R., Osborne, R. and Mitchell, J. (1979) *Brain Res.*, **178**, 467.
- Simon, J. R., Contrera, J. F. and Kuhar, M. J. (1976) *J. Neurochem.*, **26**, 141.
- Takeuchi, A. and Takeuchi, N. C. (1964) *J. Physiol.*, **170**, 296.
- Tan, C. H. and Abkowitz, S. J. (1979) *Neurosci. Lett.*, **14**, 339.
- Taraskevich, P. S. (1975) *J. Gen. Physiol.*, **65**, 677.
- Usherwood, P. N. R. (1972) *J. Physiol.*, **227**, 527.
- Van der Berg, C. J., Natheson, D. F., Ronda, G., Reijnierse, G. L. A., Blokhuis, G. C. D., Kroon, M. C., Clarke, D. D. and Garfinkel, D. (1975) in *Metabolic Compartmentation and Neurotransmission. Relation to Brain Structure and Function* (eds S. Berl, D. D. Clarke and D. Schneider) (New York: Plenum Press) p. 515.
- Warner, S. J. C., Cantrill, R. C. and Brennan, M. J. W. (1981) *Life Sci.*, **28**, 163.
- Wheeler, D. D. (1978) *J. Neurochem.*, **30**, 109.
- Wheeler, D. D. (1979) *J. Neurochem.*, **33**, 883.
- Whittaker, V. P. (1968) *Biochem. J.*, **106**, 412.
- Whittaker, V. P., Dowdall, M. J. and Boyner, A. F. (1972) *Biochem. Soc. Symp.*, **36**, 49.
- Yoshida, K. and Imura, H. (1979) *Brain Res.*, **172**, 453.
- Zingde, S., Rodrigues, V., Joshi, S. M. and Krishnan, K. S. (1983) *J. Neurochem.*, **41**, 1243.



ASHWINI DESHPANDE, S. F. D'SOUZA and G. B. NADKARNI

Food Technology and Enzyme Engineering Division, Bhabha Atomic Research Centre, Bombay 400 085, India

**Abstract.** *Trigonopsis variabilis* induced for D-amino acid oxidase and catalase was immobilized by entrapment in polyacrylamide beads obtained by radiation polymerisation. Permeabilization of the cells was found to be essential for optimal activity of the enzymes in free cells. However, the process of entrapment itself was found to eliminate the permeability barrier of cells immobilized in polyacrylamide. The two enzymes exhibited a differential response on polyacrylamide entrapment. Thus, D-amino acid oxidase activity was stabilized to heat inactivation whereas catalase in the same cells showed a destabilization on entrapment in polyacrylamide. The coimmobilized enzyme preparation showed an operational half life of 7-9 days after which the D-amino acid oxidase activity remained stable at a value 35-40% of that of the initial activity for a study period of 3 weeks. Coimmobilization of  $MnO_2$  was not effective in enhancing the operational life of the enzyme preparation.

**Keywords.** Coimmobilization; D-amino acid oxidase; *Trigonopsis variabilis*; polyacrylamide beads.

## Introduction

Recently keto acid therapy has been found to be useful in the management of chronic uremia (Walser, 1978; Adibi *et al.*, 1984). The practical use of this therapy, however, has been limited due to the lack of suitable methods for the production of the keto analogues of all amino acids. Enzymatic methods now investigated for the preparation of keto acids include the use of either L or D-amino acid oxidases (Wikström *et al.*, 1982; Szwajcer *et al.*, 1982). The use of D-amino acid oxidase [D-amino acid: oxygen oxidoreductase (deaminating); EC 1.4.3.3] has the advantage of simultaneous separation of the natural L-isomer from chemical DL-racemates along with the keto acids.

Of the several micro-organisms screened, the yeast *Trigonopsis variabilis* has been found to be one of the most potent sources of D-amino acid oxidase which could act on most of the amino acids (Brodelius *et al.*, 1981; Kubicek-Pranz and Röhr, 1985). Earlier studies have shown the feasibility of immobilizing this enzyme in Ca-alginate using intact cells (Brodelius *et al.*, 1981). However,  $H_2O_2$ , the by-product of D-amino acid oxidase has been found to inhibit the enzyme as well as decarboxylate the keto acid thus resulting in low operational stability. Several methods directed at the improvement of the operational stability include coimmobilization of  $H_2O_2$  degrading agents like  $MnO_2$  and activated charcoal (Brodelius *et al.*, 1981; Szwajcer *et al.*, 1982). In the present studies, the yeast *T. variabilis* grown under conditions optimum for the induction of D-amino acid oxidase were also found to contain large excess of catalase which could not only be useful in detoxifying  $H_2O_2$  but can simultaneously release utilizable oxygen for the D-amino acid oxidase reaction.

## Materials

Acrylamide, N,N'-methylene-bis-acrylamide (Bis) and DL-methionine were obtained from Sisco Research Laboratories, Bombay. The various other DL-amino acids were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Other chemicals were obtained from standard sources.

## Culture conditions and induction of enzymes

The strain *T. variabilis* NCIM 3344 was obtained from the National Chemical Laboratory, Pune. It was maintained by a biweekly transfer on MGYP agar slants containing malt extract—0.2%, glucose—1%, yeast extract—0.3%, peptone—0.5% (pH 6.4 to 6.8) in 2% agar. For the induction of D-amino acid oxidase, the yeast was grown aerobically for 3 days in a liquid medium containing 0.3% DL-methionine (Berg and Rodden, 1976). The cells were harvested by spinning in cold (0–4°C) at 8,000 *g* for 5 min and washed twice with cold isotonic saline. The cells grown under these conditions were also found to contain high levels of catalase.

## Permeabilization of yeast cells

To 10 ml of 10% cell suspension, 5 mL chloroform and 2 mg Na-deoxycholate were added and the mixture was incubated at 37°C for 10 min with intermittent stirring. The cells were then washed free of the permeabilizing agents with cold saline and resuspended in saline to a 50% cell concentration. Unless otherwise stated, these cells are referred to as the treated cells. The amount of cells used in these studies has been indicated as wet weight of packed cells.

## Enzyme assays

D-Amino acid oxidase was assayed by stirring 100 mg of yeast cells (immobilized or otherwise) in 5 ml of 50 mM DL-methionine contained in 50 mM pyrophosphate buffer (pH 8.5) for 30 min at room temperature. The reaction was terminated by heating in a boiling water bath for 10 min, the cells sedimented by centrifugation and the  $\alpha$ -keto acids in the supernatant estimated using dinitrophenylhydrazine reagent (Brodelius *et al.*, 1981). One unit of D-amino acid oxidase activity has been defined as  $\mu$  mol of  $\alpha$ -keto acid formed per hour.

Catalase activity was assayed by the method described earlier (D'Souza and Nadkarni, 1980). One unit of catalase activity has been defined as  $\mu$  mol of  $\text{H}_2\text{O}_2$  decomposed per min.

## Immobilization of yeast cells in polyacrylamide beads

An aqueous solution of acrylamide (20%), Bis (0.8%) and yeast cells (10% w/v) was

suspended in 50 mM pyrophosphate buffer (pH 8.5) and stored at 0–4°C.

## Results

### Effect of pretreatment on enzyme activities of yeast cells

D-Amino acid oxidase in *T. variabilis* is an intracellular enzyme and exhibits a permeability barrier for the substrate. Different methods were investigated for the permeabilization of the yeast cells. It can be seen from table 1 that D-amino acid oxidase activity of yeast cells increases upto 10-fold on treatment with permeabilizing agents. Unlike D-amino acid oxidase, considerable amount of catalase activity in the same cells could be detected in whole cells even though for expression of total catalase activity, permeabilization of the cells was found to be essential. Catalase activity of yeast cells also increased from 2,800–15,400 units/g on treatment with chloroform-Na-deoxycholate which were routinely used for permeabilizing the cells.

**Table 1.** Permeability of yeast cells.

Permeabilizing agent	Activity (u/g)
None	19
Toluene (5% v/v)	107
Toluene (50% v/v)	152
Toluene + Na-deoxycholate (25 mg%)	194
Chloroform (50% v/v)	188
Chloroform + Na-deoxycholate	200
Sonication	85
Freeze-thawing	240

The cells were permeabilized with organic solvents as described in the text. Cell suspension (50%) was sonicated in an MSE ultrasonic disintegrator model 60 W at 17–20 KC for 15 min at 4°C. Freezing and thawing was carried out by rapidly freezing the yeast cells suspensions (50%) in saline in acetone solid CO<sub>2</sub> mixture and thawing in tap water. This process was repeated 12 times.

The yield of D-amino acid oxidase and catalase activities in various cell preparations after entrapment are presented in table 2. Intact cells on entrapment showed greater yield of D-amino acid oxidase activity as compared to the permeabilized entrapped cells. Although permeabilized cells showed a 5–6-fold increase in catalase activity, the yield of catalase activity on entrapment of these cells was comparable to that of the untreated entrapped cells. These results suggest that permeabilization is not essential for expression of optimal activities of both the enzymes on immobilization, as the process of immobilization alters the permeability barrier. Thus in further studies, non-permeabilized (intact) cells have been used for immobilization.



**Table 2.** Yield of enzyme activity on entrapment.

	D-Amino acid oxidase activity of yeast cells ( $\mu\text{mol/h/g}$ of cells)	Catalase activity of yeast cells ( $\mu\text{mol/min/g}$ of cells)
None (intact cells)	5	2,800
Permeabilized	200	15,400
Intact cells + entrapment	240	2,150
Permeabilized cells + entrapment	180	2,380

The cells were permeabilized using chloroform and Na-deoxycholate as stated in the text. The units of activity are expressed in the text.

### *Optimal conditions for polyacrylamide entrapment*

The immobilized preparation with optimum D-amino acid oxidase activity was obtained when a mixture containing 20% acrylamide and 0.8% Bis was exposed to a radiation dose of 400 kR. The scanning electron micrograph of the lyophilized cross-sectioned bead showed a porous, spongy infrastructure. The D-amino acid oxidase activity varied inversely with the bead diameter (figure 1). Beads with an average diameter of 2 mm were used in the studies. The cells immobilized in polyacrylamide beads obtained under optimal conditions also retained considerable amount of catalase activity. Catalase activity of these preparations was about 500 times more than that of D-amino acid oxidase.

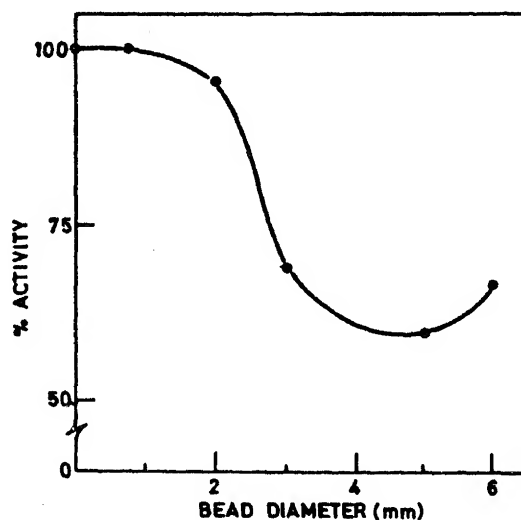
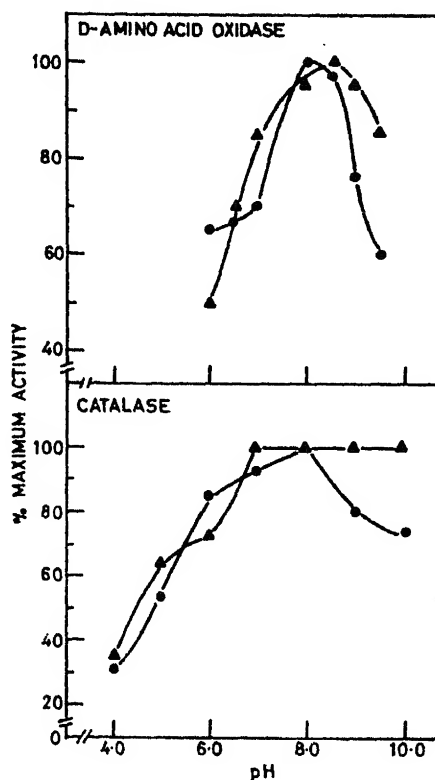


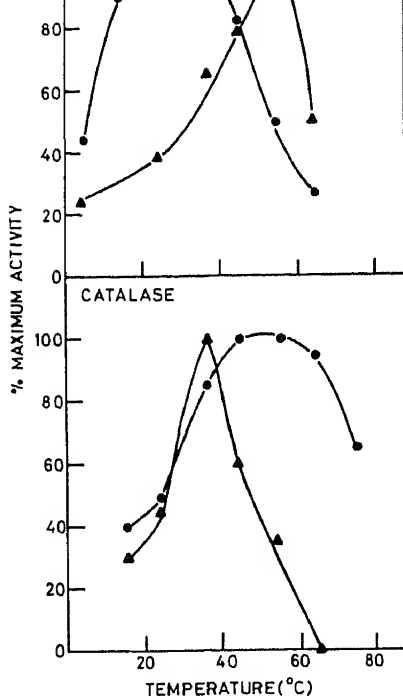
Figure 1. Effect of bead diameter on D-amino acid oxidase activity of immobilized cells.

The kinetic and stability characteristics of the enzymes in immobilized cells were compared to those of the free cells. D-amino acid oxidase in both the preparations was optimally active between pH 8.0–8.5. At this pH, the coimmobilized catalase also showed retention of 90% of its optimal activity (figure 2).  $K_m$  for the activity with D-methionine as substrate was found to be altered from  $3.6 \times 10^{-3}$  M to  $5.2 \times 10^{-3}$  M on entrapment.



**Figure 2.** pH activity profiles of D-amino acid oxidase and catalase. The buffers (50 mM) used were: acetate (pH 4.0–5.0) phosphate (pH 6.0–8.0) and pyrophosphate (pH 8.0–10.0). (●), Free cells; (▲), immobilized cells.

The coimmobilized enzyme preparation showed a differential response to temperature on polyacrylamide entrapment. Thus, temperature optimum of D-amino acid oxidase increased from 37°–55°C on immobilization; however, catalase in the same cells showed a decrease in the temperature optimum on entrapment (figure 3). Immobilized D-amino acid oxidase was found to be more thermostable as compared to the native cells. On the other hand, a reduction in the thermostability was obser-



**Figure 3.** Temperature activity profiles of D-amino acid oxidase and catalase. (●), Free cells; (▲), immobilized cells.

### *Operational stability*

Operational stability was studied by continuously passing 20 mM DL-methionine solution through a column containing the immobilized cell beads (5 g cells) at room temperature. For comparison, the same studies were simultaneously carried out using immobilized yeast cells containing coimmobilized  $\text{MnO}_2$  (figure 5). Both the columns showed an operational half-life of about 7–9 days. Inclusion of an additional  $\text{H}_2\text{O}_2$  degrading agent like  $\text{MnO}_2$  failed to prolong the operational half-life even though the initial rates of conversion in the  $\text{MnO}_2$  columns were about 1.5 times higher than those without  $\text{MnO}_2$ . The eluants from columns containing  $\text{MnO}_2$  beads were coloured. Both the columns retained a constant conversion rate of about 35–40% of the original activity for a study period of 3 weeks.

### **Discussion**

*T. variabilis* cells grown under aerobic conditions for the induction of D-amino acid oxidase were also found to induce high amounts of catalase. D-amino acid oxidase of *T. variabilis* is intracellular. Catalase from the same cells showed a part of its activity with the whole cells (patent catalase), whereas a major part was found to be intracellular (cryptic catalase). Such a distribution of catalase is known to occur in *Saccha-*

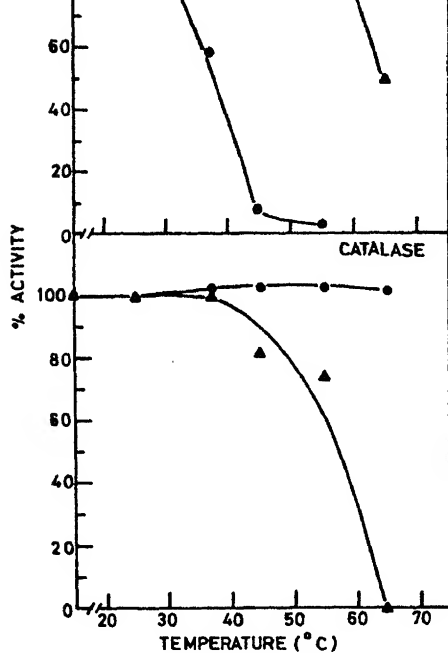


Figure 4. Thermostability of D-amino acid oxidase and catalase. The cell suspension was subjected to the desired temperature for 20 min and 10 min for D-amino acid oxidase and catalase respectively followed by rapid cooling by immersion in ice. Residual enzyme activity was determined. (●), Free cells; (▲), immobilized cells.

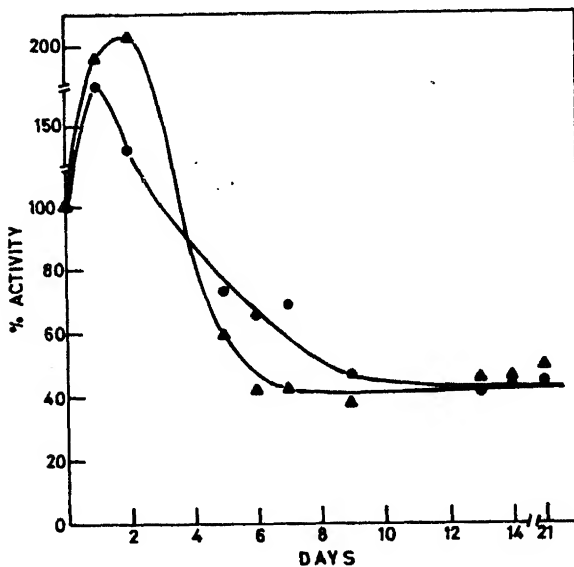


Figure 5. Operational stability of immobilized *T. variabilis*. (●), Without  $MnO_2$ ; (▲), with  $MnO_2$ .

1981), present observations indicate that permeabilization of cells was very essential for expression of optimal D-amino acid oxidase and catalase activities in native cells even though permeabilization was not found to be essential for entrapment in polyacrylamide. Entrapment in polyacrylamide is known to bring about changes in the permeability barrier (D'Souza and Nadkarni, 1980; Godbole *et al.*, 1980). However, unlike polyacrylamide, entrapment in various other supports like Ca-alginate, agar, agarose, hen egg white etc. required permeabilized cells for expression of D-amino acid oxidase (unpublished work).

Even though the pH compatibility of both enzymes was satisfactory, one of the notable characteristics of the coimmobilized system was the differential response of the two enzymes in the same cells to temperature. The D-amino acid oxidase of *T. variabilis* was stabilized to heat inactivation whereas catalase in the same cells showed a destabilization on entrapment in polyacrylamide. Similar destabilization of catalase in yeast *S. cerevisiae* entrapped in polyacrylamide has also been reported (D'Souza and Nadkarni, 1980).

One of the limitations reported so far for the use of immobilized D-amino acid oxidase for large scale operation has been the low operational stability which ranges from a few hours to about 5 days. The reasons for the comparatively short half lives of D-amino acid oxidase observed in Ca-alginate has been mainly attributed to the inactivating effect of  $H_2O_2$ . In addition, however the low intrinsic stability of D-amino acid oxidase itself may be responsible for the elicitation of a short half life since efficient combination of  $H_2O_2$  degrading agents like  $MnO_2$  and catalase failed to improve the operational stability in the present studies. At present, we are identifying the factors responsible for the instability of the system in order to enhance the operational stability.

## References

- Adibi, S. A., Fekl, W., Langenbeck, U. and Schauder, P. (eds) (1984) in *Branched chain amino and keto acids in health and disease* (Basel: Karger) p. 1.
- Berg, C. P. and Rodden, F. A. (1976) *Anal. Biochem.*, **71**, 214.
- Brodellius, P., Nilsson, K. and Mosbach, K. (1981) *Appl. Biochem. Biotechnol.*, **6**, 293.
- D'Souza, S. F. and Nadkarni, G. B. (1980) *Biotechnol. Bioeng.*, **22**, 2191.
- Godbole, S. S., D'Souza, S. F. and Nadkarni, G. B. (1980) *Enzyme Microb. Technol.*, **2**, 223.
- Kaplan, J. G. (1963) *J. Gen. Physiol.*, **47**, 103.
- Kawashima, K. and Umeda, K. (1976) *Agric. Biol. Chem.*, **40**, 1143.
- Kubicek-Pranz, E. M. and Röhr, M. (1985) *Biotechnol. Lett.*, **7**, 9.
- Szwajcer, E. M., Brodellius, P. and Mosbach, K. (1982) *Enzyme Microb. Technol.*, **4**, 409.
- Walser, M. (1978) *Am. J. Clin. Nutr.*, **31**, 1756.
- Wikström, P., Szwajcer, E., Brodellius, P., Nilsson, K. and Mosbach, K. (1982) *Biotechnol. Lett.*, **4**, 153.

J. K. DEB\*, R. GOYAL and M. CHAKRAVORTY†

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India

\*Present address: Department of Chemistry, Indian Institute of Technology, New Delhi 110 029, India

**Abstract.** The transport of  $\alpha$ -methyl-D-glucoside and two aminoacids, L-phenylalanine and L-leucine by a temperature sensitive fatty acid requiring mutant of *Salmonella typhimurium* was studied under conditions of supplementation with *cis* or *trans*-unsaturated fatty acids. The results of such experiments definitely establish a relationship between the fatty acids composition of the membrane and the transport property of the cells. Cells grown in the presence of *trans*-unsaturated fatty acids cannot transport so efficiently as compared to the *cis*-unsaturated fatty acid-grown cells except linoleic acid, a *trans-trans*-unsaturated fatty acid. Protein: phospholipid ratio of the membrane also varies significantly under such conditions. The affinity of L-phenylalanine transport carrier for the substrate changes remarkably in cells grown in the presence of different *cis* or *trans*-unsaturated fatty acids and indicate the possible role of membrane lipids in membrane assembly as well as regulation of the activity of L-phenylalanine transport system.

**Keywords.** Transport; fatty acid auxotroph; *cis* and *trans*-unsaturated fatty acid.

## Introduction

The role of membrane lipids in various membrane associated biological function has become quite evident from the reports in the literature (Endo *et al.*, 1969; Milner and Kaback, 1970; Higashi and Strominger, 1970; Kundig and Roseman, 1971; Jakovcic *et al.*, 1971; Schneider and Kenedy, 1973; Silbert, 1975; Esfahani *et al.*, 1977; Mandal *et al.*, 1978; Cronan, 1978; Deb *et al.*, 1986). In most of the investigations lipid composition of the membrane has been changed by altering growth temperature and in a few cases by supplementing different fatty acids in the media. However, no direct comparison of the effect of *cis* and *trans*-fatty acid supplementation has been carried out to investigate the effect of membrane fluidity on membrane function except the results reported from our laboratory (Deb *et al.*, 1986). It has been reported from this laboratory that many of the membrane functions and especially the transport of exogenous uridine is very much dependant on the fatty acid composition of the membrane and particularly on the ratio of saturated and unsaturated fatty acids. As reported earlier (Deb *et al.*, 1986) the lipid composition of the cell membrane has been altered by supplying different fatty acids to the growth media of temperature sensitive fatty acid biosynthetic mutant, fabB2 of *Salmonella typhimurium* which cannot synthesise fatty acid at 37°C and thus requires exogenous fatty acids for growth at non-permissive temperature (Hong and Ames, 1971). To study the effect of membrane fluidity on cellular transport processes the cells were supple-

To whom all correspondence should be addressed.

Abbreviations used:  $\alpha$ -MG,  $\alpha$ -Methyl-D-glucoside; MM, minimal medium; DPH, 1,6-diphenyl-1,3,5-extriene; ANS, 1,8-anilino naphthalene sulphonic acid.

mented with either *cis* or *trans*-unsaturated fatty acid. The fatty acids used in the study were so chosen that they vary in chain length, degree of unsaturation and steric configuration (table 1). The cellular transport process being one of the most well characterised membrane functions, the transport of 3 substances,  $\alpha$ -methyl-D-glucoside ( $\alpha$ -MG), L-leucine and L-phenylalanine was chosen for study. Transport of the different solutes was studied to explore whether different transport carriers require different lipid environment.

**Table 1.** Fatty acids used.

Common name	Systematic name	No. of carbon atoms	Formula
Palmitoleic	<i>cis</i> - $\Delta^9$ -Hexadecenoic	16	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Palmitelaidic	<i>trans</i> - $\Delta^9$ -Hexadecenoic	16	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Oleic	<i>cis</i> - $\Delta^9$ -Octadecenoic	18	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Elaidic	<i>trans</i> - $\Delta^9$ -Octadecenoic	18	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Linoleic	<i>cis, cis</i> - $\Delta^9, \Delta^{12}$ -Octadecadienoic	18	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_6\text{COOH}$
Linolelaidic	<i>trans, trans</i> - $\Delta^9, \Delta^{12}$ -Octadecadienoic	18	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_6\text{COOH}$

## Materials and methods

### Chemicals

[ $^3\text{H}$ ]-L-Leucine (5200 mC/m mol), [ $^{14}\text{C}$ ]-L-phenylalanine (10 mC/m mol), [ $^{14}\text{C}$ ]- $\alpha$ -methyl-D-glucoside (4.5 mC/m mol) were obtained from Bhabha Atomic Research Centre, Bombay. 2,5-Diphenyloxazole (PPO) and 1,4-di (2-5-phenyloxazolyl benzene) (POPOP) were purchased from Amersham/Searle Corporation, USA. The fatty acids used in the study were from Sardary Research Laboratories, London, Ontario, Canada and obtained as generous gifts from Prof. B. D. Sanwal, Department of Biochemistry, University of Western Ontario, Canada. All other chemicals were of analytical grade.

### Bacterial strain

*S. typhimurium* fabB2, a fatty acid biosynthetic mutant, used in this study was a gift from Prof. B. Ames of the Department of Biochemistry, University of California, Berkeley, California, USA. The mutant is deficient in  $\beta$ -ketoacyl carrier protein synthetase (Hong and Ames, 1971). The strain LT2 was originally obtained from

Protein was determined by the method of Lowry *et al.* (1967). Micro-determination of protein was done using Coomassie blue as described by Bradford (1976). Bovine serum albumin was used as standard in both cases.

#### *Estimation of phospholipids*

Total phospholipids from membrane vesicles were extracted by the method of Bligh and Dyer (1959) and inorganic phosphate was estimated as per the method of Chen *et al.* (1956).

#### *Transport of $\alpha$ -MG, L-leucine and L-phenylalanine*

Transport of exogenous ( $\alpha$ -MG), [ $^{14}$ C]-L-leucine and [ $^{14}$ C]-L-phenylalanine was measured according to the method of Khandekar *et al.* (1975) with the following modifications. The fabB2 cells were grown at 37°C for 4–5 generations in MM containing 0.2% glycerol, 0.04% Brij-58 and 0.4 mM requisite fatty acid. LT2 cells used as control were also grown under similar condition but without any fatty acid. Subsequently [ $^3$ H]-leucine (10 nmol,  $5\text{--}9 \times 10^5$  counts/min) or [ $^{14}$ C]-L-phenylalanine (12 nmol,  $2.2 \times 10^5$  counts/min) or [ $^{14}$ C]- $\alpha$ -MG (100 nmol,  $1.5 \times 10^5$  counts/min) was added per ml of cell suspension for transport studies. Concentration of  $\alpha$ -MG, L-phenylalanine and L-leucine used in the incubations were the saturating amounts as determined experimentally under the present conditions. Counting was done in Mark II Liquid Scintillation Counter of Nuclear Chicago and Company or LKB Rack Beta Counter using toluene based scintillation fluid.

#### *Fluorescence polarisation studies*

Fluorescence spectra were recorded on Perkin-Elmer LS-5 Spectrofluorometer with 10 nm excitation and emission band pass. Fluorescence studies were carried out with membrane vesicles prepared from cells grown in *cis* or *trans*-unsaturated fatty acid supplemented media. 1,6-Diphenyl-1,3,5 hexatriene (DPH) was added from 2 mM solution in tetrahydrofuran to the sample to achieve a final concentration of 4  $\mu$ M so that tetrahydrofuran was less than 0.001%. For distribution of DPH between the hydrophobic and hydrophilic regions of the membrane the vesicles were incubated with DPH for 15 min at 37°C. Measurements were recorded using  $\lambda$  (excitation) = 355 nm and  $\lambda$  (emission) = 430 nm.

### **Results and discussion**

#### *Effect of different fatty acid supplementation on the fluidity of membrane*

It is well-known that the fluidity of the membrane is determined by the nature of the fatty acyl moiety of the cell membrane. To alter the fluidity of the membrane,



cells were grown in the presence of either *cis* or *trans*-unsaturated fatty acids. Fatty acid analysis of such cells indicated that cells grown in the presence of *trans*-unsaturated fatty acids possess higher proportion of unsaturated fatty acid (Deb *et al.*, 1986). Fluidity of such membranes was probed with lipid specific probe DPH. When membrane is treated with DPH, it goes to lipid bilayer and the value of fluorescence polarisation of DPH ('*P*') reflects the total fluidity of the membrane. Results presented in table 2 clearly indicate that in case of each pair of *cis* and *trans*-isomer (except that of linoleic and linolelaidic acid grown cells) the fluidity of *trans*-fatty acid supplemented membrane is less than those of the *cis* unsaturated fatty acid supplemented cells (as evident from higher polarisation value). Membrane vesicles from cells grown in the presence of either linoleic or linolelaidic acid exhibited similar fluorescence polarisation value. This is in conformity with the physical properties of the fatty acids used to supplement the media. Preliminary experiments with 1,8-anilino naphthalene sulphonic acid (ANS) also support the results obtained with DPH (ANS data not presented, will be published in detail later).

**Table 2.** DPH polarisation values of the membrane vesicles of fabB2 supplemented with different fatty acids.

Fatty acid supplementation	Temperature °C	' <i>p</i> ' (polarisation value)
—	30	0.27
Oleic	37	0.23
Elaidic	37	0.32
Palmitoleic	37	0.24
Palmitelaidic	37	0.28
Linoleic	37	0.28
Linolelaidic	37	0.29

### *Effect of different cis or trans fatty acid supplementation on cellular transport process*

The effect of supplementation with *cis* or *trans*-unsaturated fatty acids on the transport of  $\alpha$ -MG, L-phenylalanine, L-leucine was studied (results of experiment have been presented in figures 1–3). In these experiments the *trans*, *trans*-unsaturated fatty acid linolelaidic acid has also been used as it resembles *cis*-unsaturated fatty acid in configuration and physical properties. In all the cases (figures 1–3) the transport as well the steady state level in *trans*-unsaturated fatty acid grown cells are invariably less than those in *cis*-unsaturated fatty acid grown cells although degree of difference varies from one transport system to another. This situation was not expected in case of linoleic-linolelaidic acid pairs (figures 1C, 2C and 3C) as linolelaidic acid resembles *cis*-unsaturated acid in physical properties. Moreover, membrane vesicles prepared from linolelaidic acid supplemented cells are as fluid as the vesicles prepared from other *cis*-unsaturated fatty acid grown cells (table 2). Even then the transport in such cells is comparatively low. Thus for transport processes to function efficiently either proper fluidity of the membrane or correct lipid environment or both are necessary. Cells grown in the presence of elaidic acid are very inefficient in transporting phenylalanine. The intracellular concentration of the

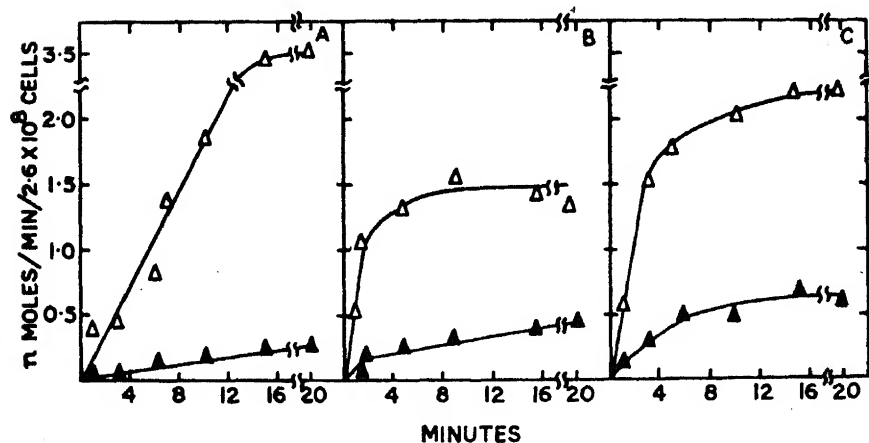


Figure 1. Transport of  $\alpha$ -methyl-D-glucoside by fabB2 grown at 37°C in media supplemented with *cis* or *trans*-isomers of 3 different fatty acids. The condition of growth was the same as that described in 'materials and methods'. *Cis* or *trans*-fatty acids used were either palmitoleic or palmitelaidic (A); oleic or elaidic, (B); and linoleic or linolelaidic (C). ( $\Delta$ ), *cis*-unsaturated fatty acid; ( $\blacktriangle$ ), *trans*-unsaturated fatty acid.

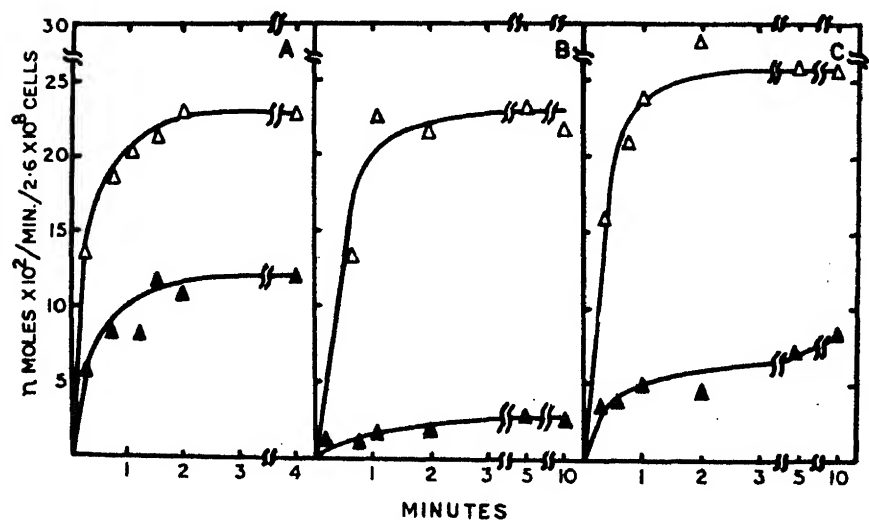


Figure 2. Transport of L-phenylalanine by fabB2 grown at 37°C in media supplemented with *cis* or *trans*-isomer of 3 different fatty acids. Experimental conditions are as described under 'materials and methods'. Symbols are as in figure 1.

(figure 2B). Our results indicate that the fluidity of the membrane is not the sole factor which determines that activity of the amino acid transport carriers.

*Effects of fatty acid composition of the cell membrane on the affinity of the L-phenylalanine transport system for phenylalanine*

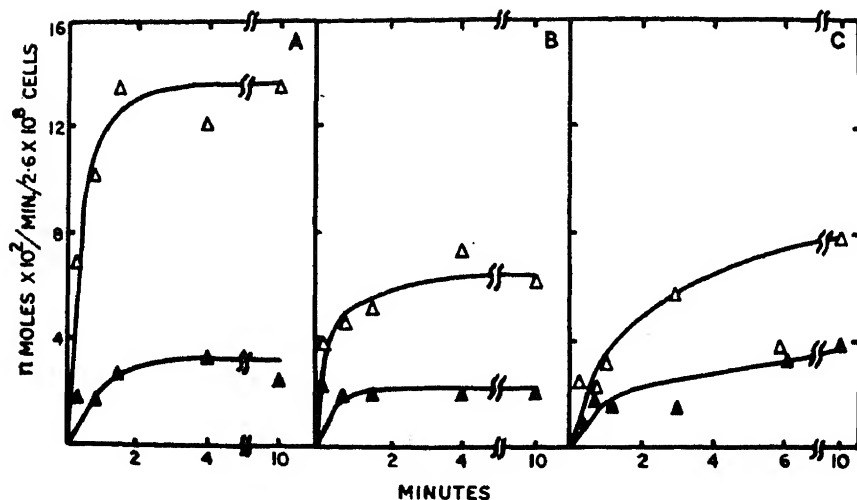


Figure 3. Transport of L-leucine by fabB2 grown at 37°C in media supplemented with *cis* or *trans* isomers of 3 different fatty acids. Experimental conditions are described under 'materials and methods'. Symbols are as in figure 1.

and phenylalanine. This may be due to inefficient functioning of the transport carriers either due to rigidity of the cell membrane or absence of specific lipids in the near vicinity of the transport carriers in the membrane or lesser number of transport carriers per cell. In order to ascertain the above, the affinity of the L-phenylalanine transport system for its substrate L-phenylalanine has been determined in cells grown in the presence of different fatty acids. The  $V_{\max}$  and  $K_m$  values for L-phenylalanine transport calculated from Lineweaver-Burk plot (figures not presented) are shown in table 3. The values for  $V_{\max}$  obtained with *cis*-unsaturated fatty acid grown cells are almost half of the values obtained with LT2 (wild) cells. The values of  $V_{\max}$  with palmitoleic and palmitelaidic (*cis-trans* pairs) acids grown cells are almost the same and half of the value of the control but the  $K_m$  varies to a great extent (almost 25 fold). The affinity of the phenylalanine transport carrier for the substrate changes remarkably in cells grown in the presence of palmitelaidic acid. This observation also confirms the idea that not only the fluidity but also the lipid composition (environment) is important in the functioning of at least some of the amino acid transport carriers. Further, the lipid environment may change the affinity of the transport carriers to their respective substrates. On the other hand, the  $K_m$  values obtained with oleic and elaidic acid grown cells (other *cis-trans* pair) are not much different while  $V_{\max}$  differs 30 fold. The elaidic acid grown cells appear to have less transport carriers than all the other types of cells. Elaidic acid grown cells cannot utilize D-leucate as the sole carbon source (unpublished results) and this observation

**Table 3.** The  $V_{\max}$  and  $K_m$  values for L-phenylalanine transport system of fabB<sub>2</sub> cells grown in the presence of different fatty acids.

Supplementation	$V_{\max}$ (n mol/min/2.6 × 10 <sup>8</sup> cells)	$K_m$
Control*	0.75	$1.5 \times 10^{-6}$ M
Palmitoleic	0.43	$8.3 \times 10^{-7}$ M
Palmitelaidic	0.40	$2.0 \times 10^{-5}$ M
Oleic	0.45	$7.7 \times 10^{-7}$ M
Elaidic	0.014	$3.3 \times 10^{-7}$ M
Linoleic	0.40	$6.6 \times 10^{-7}$ M
Linolelaidic	0.25	$6.2 \times 10^{-7}$ M

\*Rate of transport measured with LT2 cells grown in the absence of any exogenous fatty acid.

The experimental conditions (measurement of transport) were the same as described under 'materials and methods' with the modification that different concentrations of L-phenylalanine were used for different incubations. Incubation was carried out for 15 s at 25°C. The rate of transport is linear for this period.  $K_m$  for each set of cells was calculated from Lineweaver Bark plot.

**Table 4.** Lipid-protein ratio of membrane vesicles prepared from cells grown under different conditions.

Cell	Fatty acid supplementation	Growth conditions/ temperature	Protein mg/ml	Phospho- lipid mg/ml	Protein: Phospho- lipid
LT2	—	37°C	3.20	0.88	3.63:1
fabB2	—	30°C	3.8	1.26	3.01:1
fabB2	—	grown at 30°C then shifted to 37°C after 1 h of shift	3.6	2.20	1.64:1
		after 2 h of shift	1.4	0.95	1.64:1
fabB2	Oleic acid	37°C	3.0	1.01	1.47:1
fabB2	Elaidic acid	37°C	4.0	1.94	2.06:1
fabB2	Palmitoleic acid	37°C	5.1	0.70	7.28:1
fabB2	Palmitelaidic acid	37°C	5.2	1.56	3.33:1
fabB2	Linoleic acid	37°C	9.0	2.63	3.42:1
fabB2	Linolelaidic acid	37°C	6.5	2.54	2.55:1

Membrane vesicles were prepared from cells grown under different supplementation or after temperature shift. Proteins as well as phospholipids were estimated as described under 'materials and methods'.

Values given are averages of 4 to 5 preparation.

Proteins and phospholipids were estimated from same samples.

linolelaidic acid the affinity of the L-phenylalanine transport system for its substrate is equal to that of corresponding *cis*-fatty acid (linoleic acid) grown cells, but the values for  $V_{\max}$  differ.  $V_{\max}$  obtained with linolelaidic acid grown cells is 60% of the

The results presented in table 3 suggest that the membrane vesicles prepared from cells grown in media supplemented with elaidic acid demonstrate very low  $V_{\max}$  for phenylalanine transport but not much reduced  $K_m$  value as compared with that of the oleic acid supplemented cells. This suggested that cell membrane rich in elaidic acid contains less protein *per se*. Hence lipid-protein ratio of the cell membrane grown under our experimental condition was measured. Results presented in table 4 clearly indicate that the protein:lipid ratio is lower in case of *trans*-unsaturated fatty acid grown cells. This is specially remarkable in the case of elaidic acid supplemented cell. Protein:lipid ratio of the membrane vesicles grown in the presence of elaidic acid resemble very much that of the cells which have been transferred to nonpermissive temperature (37°C) from permissive temperature (30°C). It is remarkable that the two conditions as described above *i.e.* growth in the presence of elaidic acid and temperature shift are physiologically and biochemically very similar and support our observation reported earlier (Deb *et al.*, 1986). It may be discussed here that the activity of D-lactate dehydrogenase, a membrane bound enzyme also varies with variation in fatty acid supplementation and corroborates our finding on the gross lipid protein composition of the membrane. Cells grown in the presence of elaidic acid as well as cells shifted to 37°C exhibited very low D-lactate dehydrogenase activity about 1/4th to 1/10th of the activity present in LT2 strain grown at 37°C (data not given; will be published later in detail). The protein synthesis in general is not much affected in elaidic acid supplemented cells. The generation time of fabB2 in elaidic acid supplemented medium is longest. The value increases from 75 min (under palmitoleic acid supplementation, the shortest generation time) to 96 min in the presence of elaidic acid (Deb *et al.*, 1986). Such a change in the generation time cannot account for the extent of inhibition in protein synthesis in general resulting 30 fold difference in  $V_{\max}$ . The results documented above suggest that membrane lipids influence the membrane function in various ways, either by changing the affinity of membrane protein or by affecting the assembly of the proteins in the lipid matrix.

## Acknowledgements

The authors are grateful to Prof. B. D. Sanwal, Department of Biochemistry, University of Ontario, Ontario, Canada and Prof. B. Ames, Department of Biochemistry, University of California, California, USA for generous gifts of the fatty acids and the fatty acid auxotroph respectively. This investigation was supported by grants from the University Grants Commission, the Council of Scientific and Industrial Research and the Department of Science and Technology, New Delhi.

## References

- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.*, **37**, 911.
- Braford, M. M. (1976) *Anal. Biochem.*, **72**, 248.
- Chakravorty, M. (1970) *J. Virol.*, **5**, 541.
- Chen, P. S., Toribara, T. Y. and Warner, H. (1956) *Anal. Biochem.*, **28**, 1756.

- Deb, J. K., Biswas, S. K., Chakrabarti, P. and Chakravorty, M. (1986) *J. Biosci.*, **10**, 335.
- Endo, A. and Rothfield, L. I. (1969) *Biochemistry*, **8**, 3508.
- Esfahani, M., Rudkin, B. B., Cutler, C. J. and Waldron, P. E. (1977) *J. Biol. Chem.*, **252**, 3194.
- Higashi, Y. and Strominger, J. L. (1970) *J. Biol. Chem.*, **245**, 3691.
- Hoffee, P., Engelsberg, E. and Lamy, F. (1964) *Biochim. Biophys. Acta*, **79**, 337.
- Hong, J. S. and Ames, B. N. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 3158.
- Jakovcic, S., Getz, G. S., Rabinowitz, M., Jakob, H. and Swift, S. (1971) *J. Cell. Biol.*, **48**, 490.
- Kundig, W. and Roseman, S. (1975) *J. Biol. Chem.*, **246**, 1407.
- Khandekar, P. S., Burma, D. P., Taneja, S. and Chakravorty, M. (1975) *J. Virol.*, **16**, 101.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Mandal, S. B., Sen, P. C., Chakrabarti, P. and Sen, K. (1978) *Can. J. Microbiol.*, **24**, 586.
- Milner, L. S. and Kaback, H. R. (1970) *Proc. Natl. Acad. Sci. USA*, **66**, 1008.
- Ovarth, P., Schairer, H. M. and Stoffel, W. (1970) *Proc. Natl. Acad. Sci. USA*, **67**, 606.
- Schneider, E. G. and Kennedy, E. P. (1973) *J. Biol. Chem.*, **248**, 3739.
- Silbert, D. F. (1975) *Annu. Rev. Biochem.*, **44**, 315.



# Generation of bacteriophage T3 mRNAs by post-transcriptional processing by RNase III

HEMANTA K. MAJUMDER\*, SAMIT ADHYA\* and  
UMADAS MAITRA

Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, New York 10461, USA

\*Present Address: Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Calcutta 700 032, India

**Abstract.** The primary transcripts synthesized *in vitro* from a T3 DNA template by *Escherichia coli* RNA polymerase and by T3 phage-specific RNA polymerase have been characterized with regard to cleavage by RNase III and the size of the products of the cleavage reaction have been compared with those of *in vivo* T3 RNAs. It has been observed that the large RNA molecule synthesized *in vitro* by *Escherichia coli* RNA polymerase from the early region of T3 DNA are cleaved at specific sites by *Escherichia coli* RNase III to produce all the early mRNAs normally observed in T3-infected cells. In contrast, evidence presented here shows that some of the late T3 mRNAs are generated as direct products of transcription of late regions of T3 DNA by T3 RNA polymerase without mediation of RNase III, while many other late T3 mRNAs are formed by RNase III cleavage of two of the high molecular weight T3 RNA polymerase transcripts. These *in vitro* data appear to be in good agreement with the observed sizes of late T3 mRNAs formed *in vivo* in T3-infected RNase III-deficient and RNase III<sup>+</sup> *Escherichia coli* cells.

**Keywords.** RNA processing; RNase III; RNA nucleotidyl transferase; T3 promoter.

## Introduction

It is now well established that the expression of the virulent linear double-stranded DNA bacteriophages T3 and T7 in infected *Escherichia coli* cells occurs in two distinct stages. First, the early genes located in the leftmost 19–20% of the DNA (‘early’ or class I region) are transcribed by the host *E. coli* RNA polymerase, which terminates transcription at a site located at the end of the early region. The early region includes gene 1, which codes for a new phage specific RNA polymerase that transcribes the remaining 80% (‘late’ or class II and class III genes) of the phage DNA (Chamberlin *et al.*, 1970; Dunn *et al.*, 1971, 1972; Maitra, 1971; Chakraborty *et al.*, 1974; Chamberlin and Ryan, 1982). Both T3 and T7 genes are transcribed from the same strand of DNA (Summers and Siegel, 1970; Dunn *et al.*, 1972; Chakraborty *et al.*, 1974) and, according to the conventional genetic map of the phages, transcription proceeds from left to right (Summers, 1972; Beier and Hausmann, 1973; Hausmann, 1973; Studier and Movva, 1976).

In our laboratory, we have been involved in detailed characterization of various aspects of the transcription process in bacteriophage T3-infected cells. Recently, availability of a detailed restriction map of T3 DNA has allowed determination of the locations and nucleotide sequences of the major T3 RNA polymerase promoters in the T3 genome (Adhya, 1981; Adhya *et al.*, 1981; Bailey *et al.*, 1983, 1984;



region as well as a number of promoters in the class II region. These results along with the observation that there are two terminators for T3 RNA polymerase—one located at 58.5 T3 map unit while the other at 100 map unit have allowed construction of a transcriptional map for T3 RNA polymerase (Golomb and Chamberlin, 1977; Bailey and McAllister, 1980).

*In vitro*, T3 RNA polymerase, upon transcribing its specific template T3 DNA, yields 8 major discrete RNAs (designated I-VIII) with molecular weight ( $M_r$ ) of approximately 6.2, 4.7, 4.0, 2.8, 1.8, 0.9, 0.52 and  $0.21 \times 10^6$ , respectively (Chakraborty *et al.*, 1977; Golomb and Chamberlin, 1977). Comparison of the sizes of the *in vitro* T3 RNA polymerase transcripts with *in vivo* late T3 mRNAs indicates that several late mRNAs produced in T3-infected cells do not correspond to any *in vitro* T3 RNAs, and no RNAs as large as the 3 largest *in vitro* RNA species I, II and III are observed in T3-infected cells (Chakraborty *et al.*, 1977). In contrast, inclusion of purified *E. coli* RNase III in T3 RNA polymerase reaction mixtures resulted in the generation of shorter RNA products similar in size to those synthesized *in vivo* (Majumder *et al.*, 1977). These results suggested that RNase III is involved in the generation of late T3 mRNAs, but did not answer the question whether all or some of the transcripts are processed by RNase III.

In the present communication, we have carried out a systematic study of the effect of RNase III on each of the T3 RNA polymerase transcripts. The results indicate that only the high  $M_r$  transcripts, I, II, III as well as transcript VIII are processed by RNase III while species, V, VI and VII are not cleaved by RNase III under any ionic conditions. The effect of RNase III on *E. coli* RNA polymerase products of T3 DNA in the generation of T3 early mRNAs has also been investigated.

## Experimental procedures

### Enzymes

Homogeneous T3 RNA polymerase was isolated as described (Chakraborty *et al.*, 1973). *E. coli* RNA polymerase holoenzyme was purified by a modification of the published procedure (Maitra and Hurwitz, 1967). A unit of either polymerase activity is equivalent to 1 nmol of UMP incorporated in 15 min at 37°C under conditions previously specified (Chakraborty *et al.*, 1973; Maitra and Hurwitz, 1967). One picomole of T3 RNA polymerase is equivalent to 15 units. Both preparations were free of detectable RNase (including RNase III), DNase and nucleoside triphosphatase activities by criteria described previously (Chakraborty *et al.*, 1973). RNase III was prepared from *E. coli* MRE-600 by a modification of the published procedure (Robertson and Dunn, 1975). The modification involved further purification of the DEAE-cellulose fraction by chromatography on a DNA-agarose column and then on a phosphocellulose column followed by centrifugation in a 10–30% glycerol gradient. The final specific activity of the RNase III preparation was 60,000–70,000 units/mg of protein when assayed with poly(I):poly(C) as substrate under assay conditions described (Bishayee and Maitra, 1976). The purified enzyme was free of detectable endo and exoribonuclease activities as assayed on T3 RNA polymerase transcript band V ( $M_r 1.8 \times 10^6$ ).

Late *in vivo* [ $^{32}\text{P}$ ]-RNA was isolated from T3 phage-infected cells as follows: *E. coli* SY106 (20 ml) growing at 37°C in a low phosphate medium (Landy *et al.*, 1976) was irradiated with ultraviolet light to suppress host RNA synthesis (Rosenberg *et al.*, 1974) and then infected with phage T3 at a multiplicity of infection of 10. After 10 min of infection, carrier-free  $^{32}\text{P}_i$  (25  $\mu\text{Ci}/\text{ml}$ ) was added to the infected bacterial culture and infection was allowed to proceed for an additional 4 min, whereupon the 'pulse' was terminated by the addition of 20 mM potassium phosphate buffer, pH 6.8. The cells were then poured onto crushed ice containing 0.2 M  $\text{NaN}_3$ , 20 mM Tris-HCl, pH 7.4 and 5 mM  $\text{MgCl}_2$ . The cells were then harvested in a Sorvall centrifuge at 4°. From the compact pellet of cells, RNA was isolated by the diethylpyrocarbonate method of Summers (1970).

For preparation of *in vivo* early T3 [ $^{32}\text{P}$ ]-RNA, chloramphenicol (200  $\mu\text{g}/\text{ml}$ ) was added to bacterial cultures 5 min before infection with T3 and infection was then carried out for 8 min in the presence of  $^{32}\text{P}_i$  (100  $\mu\text{Ci}/\text{ml}$ ) in the culture medium.

*In vivo* experiments related to effects of RNase III on T3 late transcription were carried out using the following host strains: *E. coli* BL15, an  $\text{F}^-$  derivative of A19 (RNase III $^+$ ), and BL107, an  $\text{F}^-$  derivative of AB301-105 (RNase III $^-$ ), and *rnc* 105 (RNase III $^-$ ). Since wild-type T3 does not grow on these strains (derived from *E. coli* K 12), a mutant T3 phage that grows on *E. coli* K 12 was used in these experiments. Both the RNase III $^-$  host and the mutant T3 phage was kindly provided by Dr. F. W. Studier of Brookhaven National Laboratory.

#### *Preparation of in vitro T3 RNA synthesized by T3 RNA polymerase and by E. coli RNA polymerase*

Reaction mixtures (0.1 ml) contained 50 mM Tris-HCl, pH 7.8, 1 mM  $\text{MgCl}_2$ , 50 mM KCl, 4 mM dithiothreitol, 20 nmol of T3 DNA (expressed as deoxynucleotide residues), and 40 nmol each of ATP, GTP, UTP and CTP; UTP was labelled with either  $^3\text{H}$  or with  $^{32}\text{P}$  in the  $\alpha$ -phosphate position ( $2-5 \times 10^5$  cpm/nmol). The reaction was initiated by the addition of either T3 RNA polymerase (0.4-1 pmol) or *E. coli* RNA polymerase (5 pmol). After incubation at 37°C for 15 min, reactions were terminated by adding EDTA and sodium dodecyl sulphate (SDS) to a final concentration of 25 mM and 0.1%, respectively. Labelled RNA products were isolated by phenol extraction followed by precipitation with 2.5 vol. of ethanol at -20°C. It was then chromatographed on CFII cellulose to remove residual precursors and DNA, following the procedure of Franklin (1966) as modified by Robertson and Hunter (1975). Both the unprocessed and RNase III-processed RNAs elute from CFII cellulose at positions expected for single-stranded RNA. Purified RNAs were stored as ethanol precipitates at -20°C.

#### *Electrophoretic analysis of RNAs*

Labelled RNAs were analyzed by electrophoresis either on slab gels of 2% polyacrylamide-0.5% agarose as described (Chakraborty *et al.*, 1977; Majumder *et al.*, 1977) or in 12-cm long cylindrical gels in the presence of 6 M urea by an adaptation

of the procedure of Floyd *et al.* (1974) as described (Chakraborty *et al.*, 1977). In all electrophoretic analysis performed, [ $^{32}\text{P}$ ]-labelled 23S and 16S *E. coli* rRNA were run in a separate lane to provide  $M_r$  markers. The  $M_r$  values of all observed RNA species were estimated from their electrophoretic mobilities relative to 23S and 16S rRNA markers.

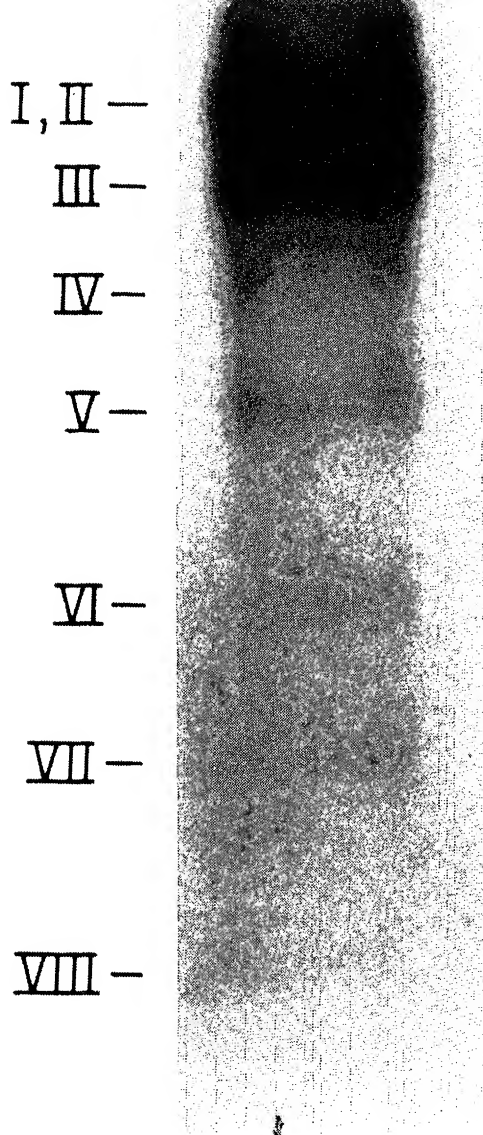
#### *Isolation of individual T3 RNA polymerase transcripts from polyacrylamide gels*

For isolation of individual T3 RNA polymerase transcripts from gels, T3 RNA polymerase transcripts were synthesized under standard reaction conditions with 2 pmol of T3 RNA polymerase and [ $\alpha$ - $^{32}\text{P}$ ]-UTP ( $2 \times 10^6$  cpm/nmol). [ $^{32}\text{P}$ ]-labelled RNA was purified as above, then subjected to electrophoresis for 12 h at 70 V on 2% polyacrylamide [acrylamide/bisacrylamide (20:1)/0.5% agarose slab gels ( $13.5 \times 10 \times 0.4$  cm)] as described (Majumder *et al.*, 1979). After electrophoresis, RNA bands were visualized by autoradiography, cut from the gel, and eluted electrophoretically into dialysis bags using a buffer that contained 20 mM Tris-HCl, pH 7.4, 40 mM sodium acetate, 1 mM EDTA, 0.1% SDS at 150 V for 10 h. RNAs were extracted with phenol, and precipitated with 2.5 vol. of ethanol. A typical separation of T3 RNA polymerase transcripts is shown in figure 1. Transcripts I and II, which can be separated on 6 M urea cylindrical gel (Chakraborty *et al.*, 1977), move with nearly identical mobilities in the non-denaturing gel system described here.

## Results

#### *Effect of RNase III on individual T3 RNA polymerase transcripts*

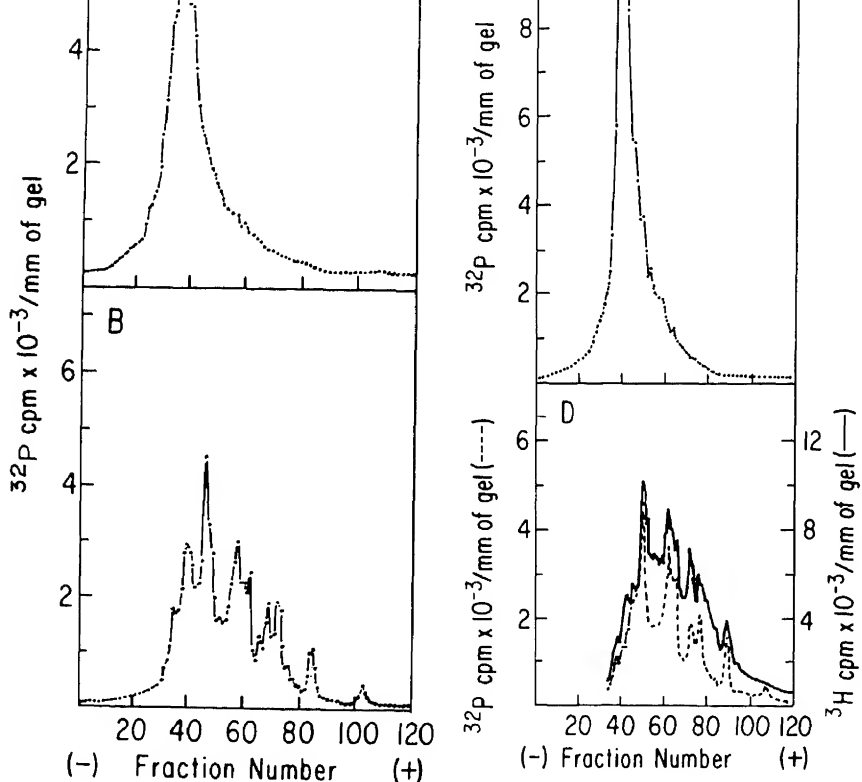
*In vitro* transcription of T3 DNA by T3 RNA polymerase yields several discrete RNA species. Polyacrylamide gel electrophoretic analysis of these products in 6 M urea in cylindrical gels resolves 8 discrete size classes of RNA, designated I-VIII, of  $M_r$ , 6.2, 4.7, 4.0, 2.8, 1.8, 0.8, 0.52 and  $0.21 \times 10^6$ , respectively (Chakraborty *et al.*, 1977; Majumder *et al.*, 1977). (2% acrylamide—0.5% agarose slab gels do not resolve species I and II as shown in figure 1). Since RNA species I, II and III formed *in vitro* were larger than any *in vivo* T3 RNA species (Majumder *et al.*, 1977), we determined whether RNase III cleaved these high  $M_r$  products. The influence of RNase III on other *in vitro* transcripts, V, VI, VII and VIII were also investigated. For this purpose, the individual T3 RNA polymerase transcripts, labelled with [ $\alpha$ - $^{32}\text{P}$ ]-UTP, were purified electrophoretically as described in 'materials and methods'. The effect of RNase III on the size of each transcript was then ascertained. As shown in figure 2, under conditions optimum for 'primary' site cleavage (Dunn, 1976) by RNase III, namely concentrations of 5–10 mM  $\text{Mg}^{2+}$  and 50–100 mM KCl in reaction mixtures, T3 RNA species (I+II) and III were cleaved by RNase III to give rise to



**Figure 1.** Autoradiogram of a polyacrylamide/agarose gel of [ $^{32}\text{P}$ ]-labelled T3 RNA polymerase transcripts. The synthesis and electrophoretic separation of T3 RNA polymerase transcripts, labelled with [ $\alpha\text{-}^{32}\text{P}$ ]-UTP, were carried out as described in 'methods'. After completion of the run, [ $^{32}\text{P}$ ]-labelled RNA species were detected by autoradiography.

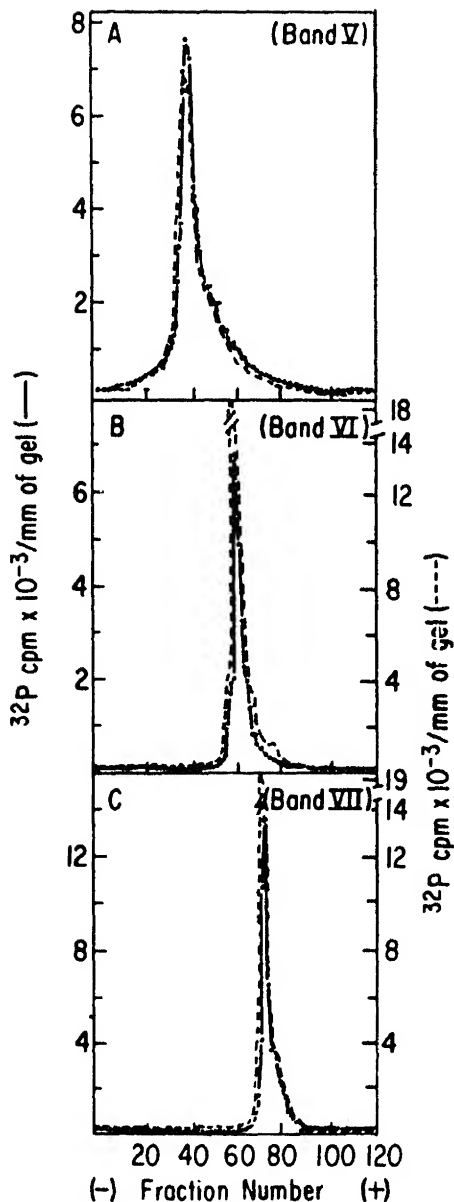
the standard T3 genetic map and share a common terminator at approximately 100% (Beier *et al.*, 1977). Thus these two RNA species transcribe overlapping transcription units.

In contrast to site specific cleavage of T3 RNA species II and III by RNase III,



**Figure 2.** Effect of RNase III on T3 RNA polymerase transcripts (I + II), and III. Reaction mixtures (50  $\mu$ l) contained 25 mM Tris-HCl buffer (pH 7.8), 5 mM  $MgCl_2$ , 100 mM KCl, 2 mM dithiothreitol, approximately 300,000 cpm of isolated [ $^{32}P$ ]-labelled T3 RNA polymerase transcript I + II (figure 2A and B) or transcript III (figure 2C and D). Reaction mixtures B and D also contained 2  $\mu$ g of RNase III. Following incubation at 37°C for 10 min, the reaction was terminated by the addition of EDTA and SDS to final concentrations of 50 mM and 0.1%, respectively, followed by dialysis against 10 mM sodium phosphate buffer (pH 6.8) for 2 h. For reaction mixtures A, B and C, an aliquot of the dialyzed material (20  $\mu$ l) containing 150,000 cpm of [ $^{32}P$ ]-radioactivity was subjected to gel electrophoresis in 12 cm long cylindrical gels by an adaptation of the procedure of Floyd *et al.* (1974) as described (Chakraborty *et al.*, 1977). Electrophoresis was carried out at 15°C at 5 mA per gel for 7 h. For reaction mixture D, a similar aliquot of the dialyzed *in vitro* RNA products was mixed with *in vivo* late [ $^3H$ ]-RNA (approximately 300,000 cpm of radioactivity) and the mixed sample was analyzed by electrophoresis as above. The gels were sliced into 1 mm fractions with an automatic Gilson gel slicer and the radioactive content of each slice was determined in 10 ml of Bray's solution in a liquid scintillation spectrometer. (A), (C), No RNase III added; (B), (D), RNase III added.

RNA species V, VI and VII were not cleaved by RNase III under the above conditions of the cleavage reaction (figure 3). T3 RNA species VIII were found to have an RNase III cleavage site giving rise to two RNA species of apparent  $M_r$  170,000 and 40,000 (data not shown). The minor T3 RNA species IV, because of low yield of recovery by elution from the gel, was not tested in the cleavage reaction.



**Figure 3.** Effect of RNase III on isolated T3 RNA polymerase transcripts V, VI and VII. Three sets of reaction mixtures, A, B and C were prepared; each set contained two separate reaction mixtures, one contained 1  $\mu\text{g}$  of RNase III while the other did not contain RNase III. Each reaction mixture (100  $\mu\text{l}$ ) was prepared as described in the legend to figure 2 except that each of the [ $^{32}\text{P}$ ]-labelled T3 RNA polymerase transcript V, VI, or VII was added as substrate for RNase III action. Following termination of the reaction, RNA products were analyzed on 12 cm cylindrical gels in 6 M urea as described in the legend to figure 2. (A), Transcript V; (B), transcript VI; (C), transcript VII; (●—●), No RNase III added; (●---●), 1  $\mu\text{g}$  of RNase III added.

Further confirmation that RNase III cleavage of the primary transcripts synthesized by T3 RNA polymerase is part of the normal pathway for the production of some of the late T3 RNAs came from analysis of *in vivo* transcripts synthesized in T3-infected host mutants deficient in RNase III. Since RNase III-deficient mutants were *E. coli* K 12 strains and wild-type T3 phage does not grow on such strains, it was necessary to use for these experiments a mutant T3 phage that grows on such strains (Studier and Movva, 1976).

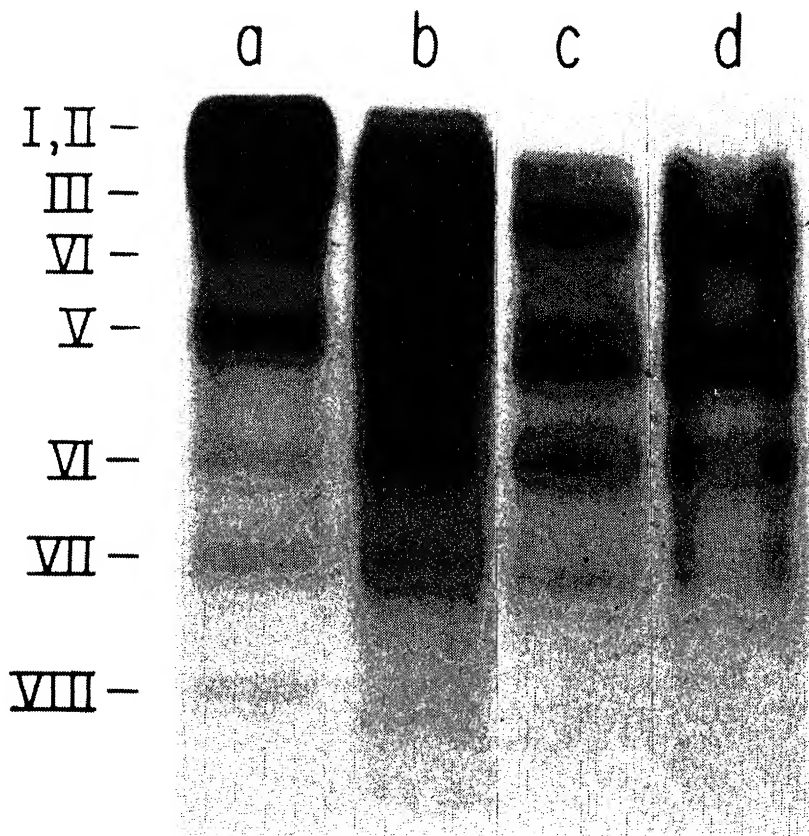
As shown in figure 4, late RNAs produced in T3-infected *E. coli* deficient in RNase III are of similar size as T3 RNA polymerase transcripts II and III. In addition, T3 RNA species V, VI and VII also have corresponding *in vivo* counterparts (figure 4, lanes a and b). Furthermore, treatment of T3 late RNAs isolated from T3-infected RNase III-deficient mutants with RNase III *in vitro* produces RNA transcripts similar in mobility to late T3 mRNAs synthesized in RNase III<sup>+</sup> cells (figure 4, lanes c and d). It should be noted that *in vitro* species VIII has no *in vivo* counterpart in RNase III<sup>+</sup> or RNase III<sup>-</sup> cells.

#### *Involvement of RNase III in the generation of T3 early mRNAs*

Early in the infection of *E. coli* by phage T3, *E. coli* RNA polymerase holoenzyme initiates transcription at one of a group of several promoters located at the genetic left end of the T3 genome to transcribe the early genes, 0.3, 0.7, 1, 1.1 and 1.3, respectively, and terminates at a site located at about 20 T3 map units (Koller *et al.*, 1974). Although termination at this site occurs quite efficiently *in vivo* (Issinger and Hausmann, 1973), *in vitro* the polymerase reads through this termination site to copy a part of the late region. The presence of *rho* protein in the *in vitro* transcription system restricts RNA polymerase to transcribing the early genes only (Dunn *et al.*, 1972; Chakraborty *et al.*, 1974; Neff and Chamberlin, 1978; Adhya *et al.*, 1979).

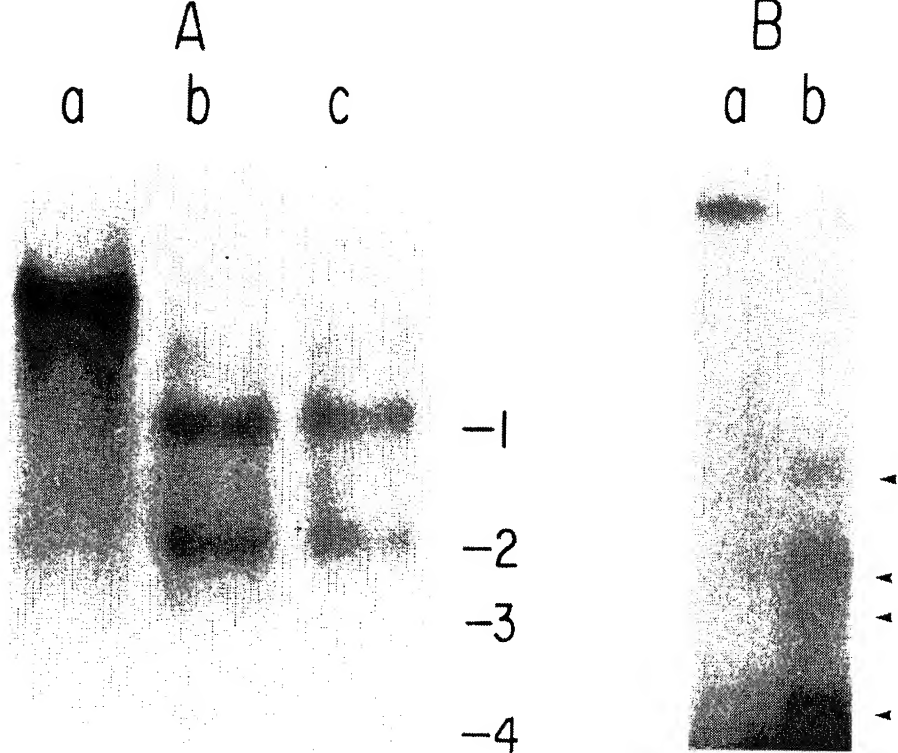
The first strong *E. coli* RNA polymerase promoter located at the left end of T3 DNA is between 0.89 and 1.69 map units, as judged by restriction fragment transcription with *E. coli* RNA polymerase (Adhya *et al.*, 1981). Initiation of transcription from this promoter and termination at 20 map units should yield a transcript of approximately  $M_r$  value  $2.5 \times 10^6$ . As shown in figure 5 (lane a), transcription of intact T3 DNA by *E. coli* RNA polymerase, under defined *in vitro* conditions, yielded a single RNA species of  $M_r$   $2.5 \times 10^6$ , corresponding to asymmetric transcription of the entire early region of T3. In contrast to the results obtained *in vitro*, analysis of the early RNAs produced after infection of *E. coli* by wild-type and deletion mutants of T3 identified 5 T3 early mRNAs (Studier and Movva, 1976; Adhya, 1981; see also figure 5, panel B). Each mRNA corresponds to each of the early genes 0.3, 0.7, 1, 1.1 and 1.3. The observed  $M_r$  values for the early RNAs are:  $1.1 \times 10^6$  for gene 1;  $0.56 \times 10^6$  for gene 0.7;  $0.41 \times 10^6$  for gene 1.3 and  $0.21 \times 10^6$  each for genes 0.3 and 1.1 (Studier and Movva, 1976).

It was of interest to determine whether, as in the case of T7 (Dunn and Studier, 1973), cleavage by RNase III is part of the pathway for manufacture of early T3 mRNAs. When RNase III was included in the *in vitro* *E. coli* RNA polymerase



**Figure 4.** T3 late RNAs made after infection of RNase III<sup>+</sup> and RNase III<sup>-</sup> hosts. BL15, an F<sup>-</sup> derivative of A19 (RNase III<sup>+</sup>), and BL107, an F<sup>-</sup> derivative of AB301-105 (RNase III<sup>-</sup>), were irradiated with UV to suppress host RNA synthesis and then infected with a mutant T3 phage that grows on *E. coli* K 12 in the presence of <sup>32</sup>P, as described in Methods. Samples were removed after 12 min of infection and [<sup>32</sup>P]-RNA was isolated by diethylpyrocarbonate method of Summers (1972). Isolated *in vivo* [<sup>32</sup>P]-RNA samples were electrophoresed on vertical slab gels alongside [<sup>32</sup>P]-labelled *in vitro* T3 RNA polymerase transcripts. In a separate experiment, an aliquot of *in vivo* [<sup>32</sup>P]-RNA isolated from T3-infected BL107 (RNase III<sup>-</sup>) cells was treated *in vitro* with 2 µg of RNase III and run in a similar slab gel. The conditions of gel electrophoresis were as described in 'methods'. After electrophoresis, gels were analyzed by autoradiography. Lane a, [<sup>32</sup>P]-labelled *in vitro* T3 RNA polymerase transcripts; lane b, T3 late [<sup>32</sup>P]-RNA isolated from RNase III<sup>-</sup> cells (BL107); lane c, T3 late [<sup>32</sup>P]-RNA from RNase III<sup>+</sup> cells (BL15); lane d, T3 late [<sup>32</sup>P]-RNA from RNase III-deficient (BL107) cells incubated with 2 µg of RNase III under standard reaction conditions. As noted above, the experiment in lane (d) was run in a separate gel. In this gel, in parallel lanes (not shown), [<sup>32</sup>P]-labelled *in vitro* T3 RNA polymerase transcripts as well as [<sup>32</sup>P]-labelled *in vivo* late mRNAs isolated from T3-infected RNase III<sup>+</sup> cells were run as controls. RNA band patterns in these control lanes were similar to those observed in lanes (a) and (c), respectively. The molecular weight values of all RNA species were calculated from their electrophoretic mobilities relative to [<sup>32</sup>P]-labelled 23S and 16S *E. coli* rRNA markers which were run in a parallel lane (not shown).





**Figure 5.** T3 early RNAs synthesized *in vitro* and *in vivo*. Labelling of RNAs is described in 'methods' while analysis of RNAs by gel electrophoresis was carried out as described in the legend to figure 4. *In vitro* RNAs, labelled with [ $\alpha$ - $^{32}$ P]-UTP, were transcribed by purified *E. coli* RNA polymerase from a T3 DNA template and subsequently purified on columns of cellulose CFII as described in 'methods'. *In vitro* transcripts thus isolated were incubated with various concentrations of RNase III for 15 min at 37° in 20 mM Tris-HCl (pH 7.8), 5 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM dithiothreitol. *In vivo* RNAs were labelled with  $^{32}$ P<sub>i</sub> after infection in the presence of chloramphenicol; the host had been irradiated with UV to suppress synthesis of host RNAs. Considerable label was incorporated *in vivo* into material of low  $M_r$  that migrates near the front. This material tends to obscure small RNAs on these gels. Panel A: *In vitro E. coli* RNA polymerase transcripts of T3 DNA: lane (a), transcript alone, no RNase III; (b) and (c), transcript + 1  $\mu$ g and 2  $\mu$ g of RNase III, respectively; Bands 1-4 represent RNA species of  $M_r$  1.25, 0.56, 0.41 and  $0.21 \times 10^6$ , respectively. Panel B: *In vivo* early RNA isolated from T3-infected *E. coli* SY106 cells labelled with H<sub>3</sub><sup>32</sup>PO<sub>4</sub> from (a) 0-4 min, (b) 0-8 min, after infection in the presence of 200  $\mu$ g/ml of chloramphenicol. The arrows represent the positions of the early RNAs. The calculated  $M_r$  values of these RNAs are 1.3, 0.6, 0.4 and  $0.21 \times 10^6$ , respectively. The intense smear at the bottom of the gel probably represents unincorporated  $^{32}$ P<sub>i</sub>.

and c). The major species correspond in size to early *in vivo* T3 mRNA (figure 5). Several minor species were also observed; these may arise by RNase III cleavage at

In the present study, we show that like the T7 system, (Dunn and Studier, 1977) the pathway for production of early T3 RNAs appears to involve endonucleolytic cleavage by RNase III of a  $2.5 \times 10^6$  large precursor RNA synthesized by *E. coli* RNA polymerase from T3 DNA template. In contrast, RNase III appears to be involved in the generation of only some of the late T3 mRNAs, while some other late T3 mRNAs are made as direct products of transcription of the late regions of T3 DNA by T3 RNA polymerase. Among the 6 major T3 RNA polymerase transcripts examined for *in vitro* cleavage by RNase III, transcripts II, III and VIII are cleaved at specific sites by RNase III. While species VIII has no *in vivo* counterpart, *in vitro* cleavage of species II and III generates RNA species that appear to co-migrate in gel electrophoresis with late mRNAs isolated from T3-infected cells. These *in vitro* data appear to be in good agreement with the *in vivo* observations that late T3 mRNAs isolated from RNase III-deficient strains contain high  $M_r$  RNA products which are of similar size as *in vitro* T3 RNA species II and III. Furthermore, the large high  $M_r$  products isolated from RNase III-deficient strains can be processed *in vitro* with purified RNase III to produce normal size T3 late mRNAs. As expected, each of the *in vitro* T3 RNA species V, VI and VII which are not cut by RNase III *in vitro*, appears to have an *in vivo* mRNA counterpart both in T3-infected RNase III<sup>+</sup> and T3-infected RNase III<sup>-</sup> cells.

In the present study the identity of *in vivo* and *in vitro* RNA products have been inferred on the basis of molecular size analysis in polyacrylamide gels. It will be important to map the RNase III cleavage sites on T3 genome and to show that *in vitro* RNA products formed by RNase III cleavage arise from the same region T3 DNA as the corresponding *in vivo* RNA. Recently, during the course of sequencing of class III T3 RNA polymerase promoters, two putative RNase III cleavage sites have been mapped around 45.0 and 64.8 T3 map units (Basu *et al.*, 1984). Furthermore, the present study has not excluded the possibility that the products of RNase III cleavage are processed further *in vivo*. It will be necessary to show that mRNAs produced *in vivo* have the same base sequences at their ends as the RNAs of similar sizes formed after RNase III cleavage.

The biological function of specific cleavage of T3 and T7 RNAs by RNase III is unknown. Unlike eukaryotic mRNAs, most prokaryotic mRNAs are polycistronic; some are monocistronic. Bacteriophage T3 and T7 mRNAs are however unique in that most of the RNAs are transcribed into a polycistronic mRNA, each from a separate transcription unit, which is subsequently cleaved at specific sites to yield mRNAs for the individual genes. Since T3 and T7 may have developed from a common ancestor, it is significant that despite the divergence of the two phage DNA sequences, RNase III cleavage sites in both the early and late mRNA sequences have been retained in both phage systems. Presumably, RNase III cleavage of polycistronic mRNAs in T3 and T7-infected cells offers some selective advantage for the phage at least on its natural hosts. Dunn and Studier (1975) have however concluded that such cleavages are not essential for growth of T7 since the phage is able to grow in RNase III-deficient strains, and all but a few T7 proteins are made at essentially the same rate in RNase III deficient as in RNase III<sup>+</sup> strains. The exceptions are the gene 0.3 protein, an early protein, and a few late proteins. Cleavage of the early

although the lysis period is somewhat lengthened in these strains as compared to RNase III<sup>+</sup> cells. These observations lend support to the view that cleavage by RNase III are not required for growth of T3 and T7 in *E. coli*. The possibility, however, exists that such cleavage may be essential for good growth of these phages in some of the hosts upon which T3 and T7 grow in nature.

## Acknowledgement

This work was supported by grants GM15399 and P30CA 13330 from the National Institutes of Health, USA.

## References

- Adhya, S. (1981) *Transcription of Bacteriophage T3 DNA Catalyzed by Escherichia coli and T3 RNA Polymerases*, Ph.D. Thesis, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York, USA.
- Adhya, S., Basu, S., Sarkar, P. and Maitra, U. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 147.
- Adhya, S., Sarkar, P., Valenzuela, D. and Maitra, U. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1613.
- Bailey, J. N., Klement, J. F. and McAllister, W. T. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2814.
- Bailey, J. N. and McAllister, W. T. (1980) *Nucleic Acids Res.*, **8**, 5071.
- Basu, S., Sarkar, P., Adhya, S. and Maitra, U. (1984) *J. Biol. Chem.*, **259**, 1993.
- Beier, H. and Hausmann, R. (1973) *J. Virol.*, **12**, 417.
- Beier, H., Golomb, M. and Chamberlin, M. (1977) *J. Virol.*, **21**, 753.
- Bishayee, S. and Maitra, U. (1976) *Biochem. Biophys. Res. Commun.*, **73**, 306.
- Chakraborty, P. R., Bandyopadhyay, P., Huang, H. H. and Maitra, U. (1974) *J. Biol. Chem.*, **249**, 6901.
- Chakraborty, P. R., Salvo, R. A., Majumder, H. and Maitra, U. (1977) *J. Biol. Chem.*, **252**, 6485.
- Chakraborty, P. R., Sarkar, P., Huang, H. H. and Maitra, U. (1973) *J. Biol. Chem.*, **248**, 6637.
- Chamberlin, M., McGrath, J. and Waskell, L. (1970) *Nature (London)*, **228**, 227.
- Chamberlin, M. and Ryan, T. (1982) *Enzymes*, **15**, 87.
- Dunn, J. J. (1976) *J. Biol. Chem.*, **251**, 3807.
- Dunn, J. J., Bautz, F. A. and Bautz, E. K. F. (1971) *Nature New Biol.*, **230**, 94.
- Dunn, J. J., McAllister, W. T. and Bautz, E. K. F. (1972) *Proc. Natl. Acad. Sci. USA*, **70**, 1559.
- Dunn, J. J. and Studier, F. W. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 3296.
- Dunn, J. J. and Studier, F. W. (1975) *J. Mol. Biol.*, **99**, 487.
- Floyd, R. W., Stone, M. P. and Joklik, W. K. (1974) *Anal. Biochem.*, **59**, 599.
- Franklin, R. M. (1966) *Proc. Natl. Acad. Sci. USA*, **55**, 1504.
- Hausmann, R. (1973) *Annu. Rev. Microbiol.*, **27**, 51.
- Golomb, M. and Chamberlin, M. (1977) *J. Virol.*, **21**, 743.
- Issinger, O. G. and Hausmann, R. (1973) *J. Virol.*, **11**, 465.
- Koller, T., Sago, J. M. and Bujard, H. (1974) *Biopolymers*, **13**, 995.
- Landy, A., Abelson, J., Goodman, H. M. and Smith, J. D. (1976) *J. Mol. Biol.*, **29**, 457.
- Maitra, U. (1971) *Biochem. Biophys. Res. Commun.*, **43**, 443.
- Maitra, U. and Hurwitz, J. (1967) *J. Biol. Chem.*, **242**, 4897.
- Majumder, H. K., Bishayee, S., Chakraborty, P. R. and Maitra, U. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4891.
- Majumder, H. K., Maitra, U. and Rosenberg, M. (1979) *Proc. Natl. Acad. Sci. USA*, **74**, 4891.
- Neff, N. F. and Chamberlin, M. (1978) *J. Biol. Chem.*, **253**, 2455.
- Robertson, H. D. and Dunn, J. J. (1975) *J. Biol. Chem.*, **250**, 3050.
- Robertson, H. D. and Hunter, T. (1975) *J. Biol. Chem.*, **250**, 418.
- Rosenberg, M., Kramer, R. A. and Steitz, J. A. (1974) *J. Mol. Biol.*, **89**, 772.
- Sarkar, P., SenGupta, D., Basu, S. and Maitra, U. (1985) *Gene*, **33**, 351.
- Studier, F. W. and Movva, N. (1976) *J. Virol.*, **19**, 136.
- Summers, W. C. (1970) *Anal. Biochem.*, **33**, 449.

# Transcriptional specificity after mycobacteriophage I3 infection

V. NAGARAJA and K. P. GOPINATHAN\*

Microbiology & Cell Biology Laboratory, Indian Institute of Science, Bangalore 560 012, India.

**Abstract.** Transcriptional regulation following mycobacteriophage I3 infection has been investigated. For this purpose, RNA polymerase mutants (*rif<sup>r</sup>*) of host bacterium, *Mycobacterium smegmatis* have been isolated and characterised. Phage growth in *rif<sup>s</sup>* and *rif<sup>r</sup>* cells in presence of rifampicin revealed the involvement of host RNA polymerase in phage genome transcription. This was confirmed by studies on *in vivo* RNA synthesis as well as by direct RNA polymerase assay after phage infection. Significant stimulation in RNA polymerase activity was seen following phage infection. The maximal levels were attained in about 60 min post infection and maintained throughout the phage development period. The stimulation of polymerase activity was most pronounced when the phage DNA was used as the template. RNA polymerases from uninfected and phage-infected *Mycobacterium smegmatis* have been purified to homogeneity. The enzyme purification was accomplished by a rapid procedure utilising affinity chromatography on rifampicin-Sepharose columns. Subunit structure analysis of the purified RNA polymerase from uninfected and phage-infected cells showed the presence of  $\alpha$ ,  $\beta$ ,  $\beta'$  and  $\sigma$  subunits similar to the other prokaryotic RNA polymerases. In addition, a polypeptide of 79,000 daltons was associated with the enzyme after phage infection. The enzymes differed in their properties with respect to template specificity. Phage I3 DNA was the preferred template for the modified RNA polymerase isolated from infected cells which may account for the transcriptional switch required for phage development.

**Keywords.** RNA polymerase; rifampicin-Sepharose; affinity chromatography; rifampicin resistance; modified RNA polymerase; transcriptional control.

## Introduction

Infection with bacteriophages brings about dramatic changes in the intracellular environment of host bacteria. These major adaptations assure specific, rapid and temporally correct transcription needed for the phage growth and multiplication. Bacteriophages are known to invoke novel transcriptional controls, thereby regulating their genome transcription (Doi 1977; Travers, 1976).

We have employed *Mycobacterium smegmatis*-phage I3 system as a model to study host-virus interaction. Mycobacteriophage I3 is a temperate, transducing phage with a growth cycle in the synthetic medium lasting for 5 h comprising of a 200 min latent period and a 60–90 min rise period (Nagaraja and Gopinathan, 1980). In conformity with the slow growing nature of their hosts, the mycobacteriophages also show prolonged growth patterns. The host DNA is not degraded after phage I3 infection until the end of intracellular development period (Nagaraja and Gopinathan, 1981). Evidently, the phage must be exerting control at the level of RNA synthesis by diverting the host transcriptional machinery towards its own transcription. This

\*To whom all correspondence should be addressed.

Abbreviations used: BSA, Bovine serum albumin; DTT, dithiothreitol; DMSO, dimethylsulphoxide; SDS,

transcriptional control has, therefore been investigated in detail. The results presented in this communication show that phage I3 is employing the host RNA polymerase for its genome transcription, after suitably modifying the enzyme. The RNA polymerase isolated from phage-infected cells differs markedly from the one isolated from uninfected bacteria in subunit structure and template specificity.

## Materials and methods

### Chemicals

Nucleoside triphosphates (ATP, GTP, CTP, UTP), calf thymus- and salmon sperm DNA, poly d(A-T), poly (G-U), rifampicin (rif), bovine serum albumin (BSA fraction V, recrystallised), dithiothreitol (DTT), dimethylsulphoxide (DMSO), sodium dodecyl sulfate (SDS) and 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) were from Sigma Chemical Company, St. Louis, Missouri, USA. [ $^3\text{H}$ ]-Uracil was from Bhabha Atomic Research Centre, Bombay. [ $^3\text{H}$ ]-UTP was from New England Nuclear, Boston, Massachusetts, USA.

### Bacteria and phage

A clear plaque forming mutant of phage I3 (Gopinathan *et al.*, 1978) was employed for all the experiments described here. The host strain used was *M. smegmatis* SN2. The bacteria and phage were grown as described elsewhere (Nagaraja and Gopinathan, 1980).

Rifampicin-resistant *M. smegmatis* cells were isolated by mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Bacteria growing in exponential phase were harvested, suspended in minimal medium containing 200  $\mu\text{g}/\text{ml}$  of NTG and incubated for 45 min at 37°C. The cells were washed free of the mutagen, inoculated into nutrient broth and were grown overnight. The drug resistant colonies were selected on nutrient agar plates containing 100  $\mu\text{g}/\text{ml}$  rifampicin.

For growth or inhibition studies, rifampicin was added to the cultures from a stock solution (4 mg/ml) in DMSO. Appropriate volume of DMSO was present in control cultures.

### Preparation of cell free extracts

The cells were washed twice with extraction buffer (20 mM Tris-HCl, pH 7.4, 5 mM  $\text{MgCl}_2$ , 5 mM  $\beta$ -mercaptoethanol and 10% glycerol) and suspended in the same buffer. Extracts were prepared by passing the cell suspension through French pressure cell (18000 psi) followed by centrifugation at 2,000  $g$  for 30 min. The supernatant was further centrifuged at 100,000  $g$  for 90 min and this S-100 supernatant was used for all the enzyme assays.

dissolved in a small volume of buffer A (10 mM Tris-HCl, pH 7.9, 1 mM MgCl<sub>2</sub>, 5 mM DTT and 5% glycerol), and gel filtered on an Ultrogel column (1.2 × 70 cm), previously equilibrated with buffer A. The individual fractions (1.2 ml) were monitored for absorbance at 280 nm and enzyme activity. The active fractions were pooled and subjected to affinity chromatography on rifampicin-Sepharose column.

#### *Preparation of rifampicin-Sepharose*

Rifampicin-Sepharose (1.2 g) was washed with 0.5 M NaCl, suspended in 3.0 ml of water and 200 μmol of rifampicin dissolved in 40% dimethylformamide was added. The pH was adjusted to 4.5–6.0, and gently shaken for 2 h maintaining the pH between 4.5–6.0. EDC (200 μmol) was added, and the shaking was continued at room temperature (20°C) for 18–20 h. The reaction was continued for another 10 h in the presence of additional 200 μmol EDC, at the end of which 100 mg of glycine was added. After shaking for 1 h, the contents were washed successively with the following: (i) 100 ml of water, (ii) 100 ml of 0.1 M sodium acetate in 0.5 M NaCl (pH 4.5), (iii) 100 ml of 2 M urea in 0.5 M NaCl, (iv) 100 ml of 0.1 M sodium carbonate in 0.5 M NaCl, pH 9.5 and (v) excess of water.

The coupled rifampicin-Sepharose (light orange-red in colour) was stored in 0.5 M NaCl at 4°C, protected from light. Rifampicin-Sepharose was packed into a column and equilibrated with buffer A containing 50 mM KCl. The coupled rifampicin-Sepharose was relatively unstable, due to the degradation of the drug. However, if stored at 4°C, the column could be used 2–3 times within a week. After each use, the column was washed alternately with low pH and high pH buffers containing 0.5 M NaCl.

#### *NA polymerase assay*

The reaction was carried out essentially as described by Burgess (1969). The assay mixture contained in 125 μl, 20 mM Tris-HCl, pH 7.9, 1 mM magnesium acetate, 5 mM EDTA, pH 7.0, 0.1 mM DTT, 200 mM KCl, 0.4 mM potassium phosphate (pH 7.5), 2.5 μg BSA, 1% glycerol, 0.4 mM each of CTP, ATP and GTP, 0.1 mM [<sup>3</sup>H]-GTP (200 μCi/μmol), 5 μg calf thymus DNA and the enzyme. Potassium phosphate was added to the assay mixture to inhibit polynucleotide phosphorylase (Burgess 1969); the presence of phosphate had no adverse effect on polymerase activity. The reaction mixtures were incubated at 37° for 5 min and 100 μl samples were processed for the determination of trichloroacetic acid-insoluble radioactivity.

One unit of enzyme activity incorporated 1 nmol of UMP in 5 min under the conditions described. Protein estimation was carried out by the method of Lowry *et al.* (1951).

#### *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions was carried out as described by Smith and Brown (1978) with slight modifications. Apart

from the regular constituents, the 5% acrylamide gels contained 5 mM  $\beta$ -mercaptoethanol and 10% glycerol in Tris-glycine (pH 8.3). After electrophoresis the gels were stained and destained as described by Fairbanks *et al.* (1971). SDS-PAGE was carried out by the method of Laemmli (1970) in 7.5% gels. The gels were scanned at 280 nm using UV-190 Shimadzu double beam spectrophotometer, to determine the number of subunits.

### Molecular weight determination

Molecular weight of the subunits was calculated based on the mobility of reference markers—albumin (bovine plasma), ovalbumin, pepsin,  $\beta$ -lactoglobulin and lysozyme.

## Results

### Isolation and characterization of *rif<sup>r</sup>* mutants of *M. smegmatis*

Experiments were designed to see whether phage I3 is employing the host RNA polymerase or coding for its own enzyme. For this purpose, RNA polymerase mutants (*rif<sup>r</sup>*) of *M. smegmatis* were isolated and characterized. All the mutants grew at 100  $\mu$ g/ml or more of rifampicin, while the growth of the parental strain was completely inhibited by less than 1  $\mu$ g/ml of rifampicin. However, the drug resistant strains showed a lag in their growth, which was even more pronounced in the presence of the drug. To rule out the possibility that the *rif<sup>r</sup>* mutation was at the level of permeability to the drug into the organism, the growth of the strains was examined in the presence of the antibiotic and 0.01% SDS. At this concentration, SDS renders the cells permeable to the drug without exerting any growth inhibitory effect by itself. All the mutants isolated in the present studies were resistant to the drug even in the presence of SDS.

To confirm that the mutation was indeed in RNA polymerase, the enzyme activity was assayed in cell-free extracts prepared from wild type and *rif<sup>r</sup>* bacteria (table 1).

Table 1. Effect of rifampicin on RNA polymerase activity.

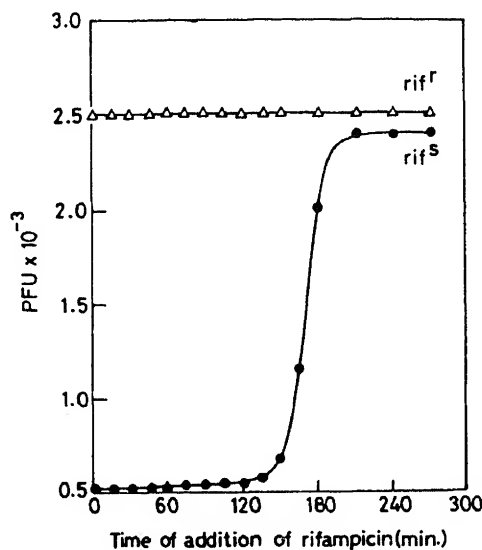
Host strain	Infection with phage I3	Enzyme activity (cpm incorporated/5 min)		
		Control (no drug)	In presence of rifampicin (100 $\mu$ g/ml)	Activity (%)
<i>rif<sup>s</sup></i>	uninfected	850	83	10
	infected	2213	163	7
<i>rif<sup>r</sup></i>	uninfected	525	550	105
	infected	1575	1560	99

The extracts were prepared from exponentially growing cells of *M. smegmatis* (uninfected or 90 min following the infection with phage I3). The S-100 fraction was used as enzyme source. The RNA polymerase reaction was carried out as described under methods. The enzyme was

lated from the mutant cells, suggesting that the mutation was at the level of RNA polymerase.

*phage is utilizing host RNA polymerase for its transcription*

To find out whether the phage is dependent on the host RNA polymerase or coding for its own enzyme, the effect of rifampicin on phage production was examined. Wild type and *rif<sup>r</sup>* cells were infected with phage I3 and the production of phage at the end of 1 cycle of growth in the presence of rifampicin (added at different times), was monitored (figure 1).



**Figure 1.** Effect of rifampicin on phage production.

*M. smegmatis*, *rif<sup>s</sup>* and *rif<sup>r</sup>* strains were infected with phage I3. The infected samples were exposed to 100 µg/ml of rifampicin at different periods during growth and the growth continued till the end of 1 cycle (300 min). The phage titers were determined at the end of one cycle of phage growth. The *rif<sup>r</sup>* mutant used in this and all the subsequent experiments was resistant upto 500 µg/ml of the antibiotic. (●), *rif<sup>s</sup>*, (Δ), *rif<sup>r</sup>*.

Phage production was completely inhibited in wild type cells when rifampicin was added at any time during the phage development period (up to 160 min, in nutrient broth). On the contrary, no inhibition of phage growth was observed in *rif<sup>r</sup>* cells, irrespective of the presence of drug. These results implied that in sensitive cells, inhibition of host RNA polymerase has resulted in the inhibition of transcription from phage genome. Phage production was not affected in the *rif<sup>r</sup>* cells in the presence of rifampicin due to insensitivity of the enzyme to the drug.

To establish this point firmly, the *in vivo* RNA synthesis was also examined in *rif<sup>s</sup>* and *rif<sup>r</sup>* cells after phage infection. [<sup>3</sup>H]-Uracil incorporation into RNA was completely blocked by rifampicin in wild type cells (figure 2). The extent of inhibition of RNA synthesis in the *rif<sup>r</sup>* mutant was much less pronounced. The reduced level of



**Table 2.** RNA polymerase purification from *M. smegmatis*\*.

Step	Total protein (mg)	Total activity (nmol UMP incorporated/5 min)	Specific activity (nmol UMP incorporated/5 min/mg protein)	Fold purification
Crude (S-100) fraction	142.10	71.36	0.50	
30–50% Ammonium sulfate fraction	35.36	35.77	1.01	2
Pooled Ultrogel fraction	6.56	173.60**	26.40	53
Rifampicin-Sepharose fraction	0.085	11.08	130.35	260

*M. smegmatis* in exponential phase ( $1 \times 10^8$  cells/ml) was used for the preparation of enzyme.

\*From 12 g wet weight of cells.

\*\*The total activity at this stage was considerably more than the earlier steps, due to the removal of inhibitory materials.

**Table 3.** RNA polymerase purification from phage I3-infected *M. smegmatis*\*.

Step	Total protein (mg)	Total activity (nmol UMP incorporated/5 min)	Specific activity (nmol UMP incorporated/5 min/mg protein)	Fold purification
Crude (S-100) fraction	139.20	180.80	1.30	
30–50% Ammonium sulfate fraction	33.32	56.62	1.70	1.3
Pooled Ultrogel fraction	6.32	291.18**	46.10	36.0
Rifampicin-Sepharose fraction	0.086	17.97	208.95	161.0

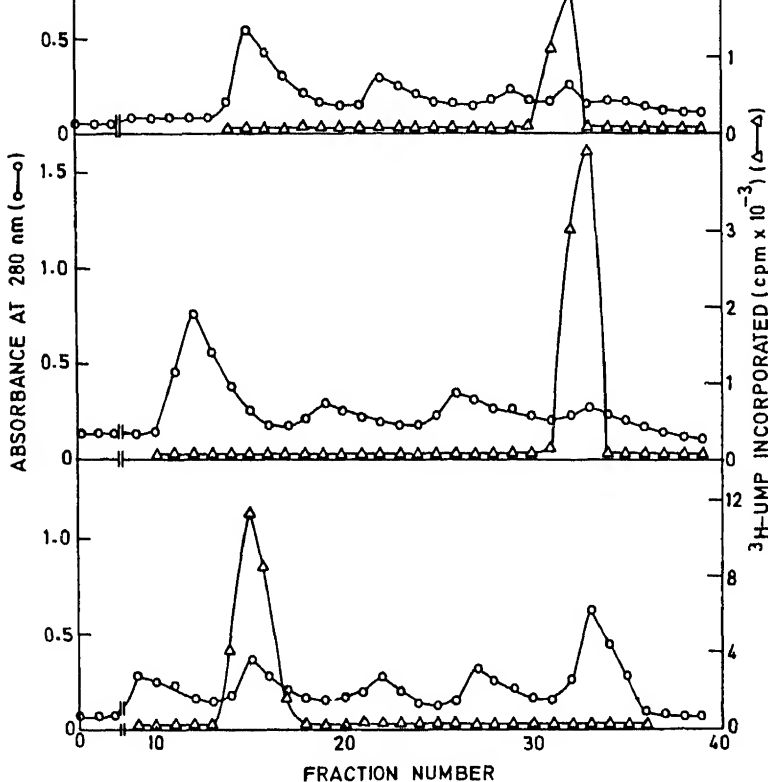
*M. smegmatis*, in exponential phase was infected with phage I3 at a multiplicity of infection of 15 in presence of 1 mM  $\text{CaCl}_2$  and incubated for 90 min at 37°C on a shaker before the cells were harvested for the isolation of the enzyme.

\*From 12 g wet weight of cells.

\*\*The total activity at this stage was considerably more than the earlier steps, due to the removal of inhibitory materials.

RNA polymerase from uninfected cells showed 3 bands corresponding to molecular weight of 151,000, 95,500 and 45,500. These bands were tentatively identified as  $\beta\beta'$ ,  $\sigma$  and  $\alpha$  subunits by analogy to the molecular weight of known prokaryotic RNA polymerases. The enzyme from infected cells had an extra band of molecular weight 79,000 in addition to other subunits. The total molecular weights of the RNA polymerases based on an  $\alpha_2\beta\beta'\sigma$  subunit structure correspond to 488,500 and 564,500 respectively, from uninfected and phage I3-infected *M. smegmatis* (cf. total molecular weight of *E. coli* RNA polymerase is 493,000, Burgess, 1969).

The pure enzymes from *M. smegmatis* (uninfected or phage I3-infected), were 10 times more sensitive to inhibition by rifampicin than the *E. coli* enzyme ( $4 \times 10^{-8}$  M



**Figure 4.** Affinity chromatography of RNA polymerases on rifampicin-Sepharose column.

Protein fractions from Ultrogel filtration step showing enzyme activity were pooled and loaded on to the affinity column. After washing the column extensively with buffer A containing 0.1 M KCl, the enzyme was eluted using a step gradient of KCl (0.2–1.2 M) in buffer A. One ml fractions were collected and assayed for enzyme activity.

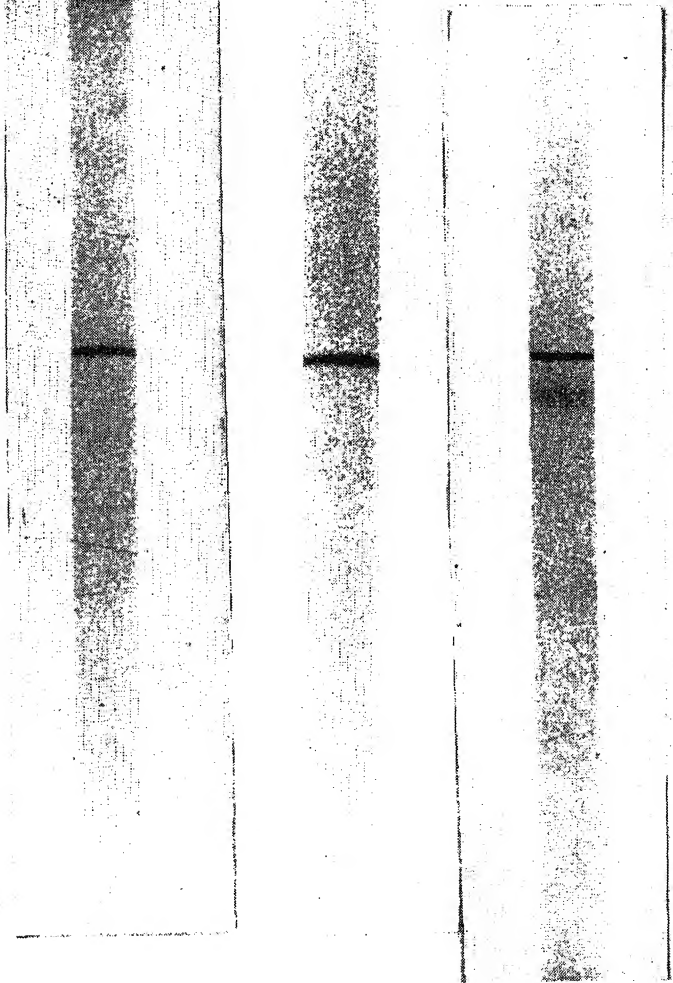
Top panel: RNA polymerase from uninfected *M. smegmatis*.

Middle panel: RNA polymerase from phage I3 infected *M. smegmatis*.

Bottom panel: RNA polymerase from *E. coli*.

### Template specificity

To check whether the association of the extra polypeptide with RNA polymerase could account for changes in transcriptional specificities, the template recognition by the modified and the parental enzymes was compared (table 2). The enzyme from phage-infected cells transcribed phage I3 DNA to a much greater extent than all other template DNAs tested. Calf thymus- and salmon sperm DNAs also served as good templates for this enzyme, while the host DNA turned out to be poor template. On the other hand, no such specificity was observed for the RNA polymerase from uninfected cells. Single stranded DNA or synthetic ribopolymers were very poor templates for both the enzymes.

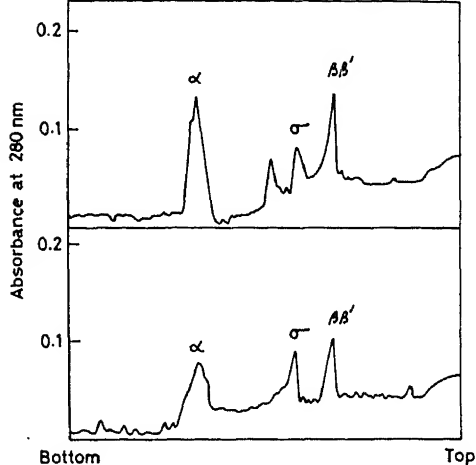


**Figure 5.** PAGE of RNA polymerases.

The electrophoresis was carried out in 5% gels at pH 8.8; 10  $\mu$ g of enzyme after rifampicin-Sepharose affinity chromatography, was used in each gel. From left to right, RNA polymerases from uninfected- and phage I3-infected *M. smegmatis*, and *E. coli*.

## Discussion

When bacteria are infected with phage, often a metabolic shift takes place channelling the macromolecular syntheses towards the phage development. Involvement of RNA polymerase in the regulation of transcriptional switch has been well documented and is primarily responsible for metabolic alterations in certain cases (Losick and Chamberlin, 1976; Doi, 1977). Different bacteriophages have evolved varying means of transcriptional control. Transcription of  $\lambda$  genome is regulated by antitermination phenomenon (Adhya *et al.*, 1976). Extensive changes have been



**Figure 6.** Subunit structure of RNA polymerase.

The purified enzymes were subjected to electrophoresis on 7.5% polyacrylamide gels under denaturing conditions. After electrophoresis the gels were scanned at 280 nm.

Top panel: RNA polymerase from phage-infected *M. smegmatis*.

Bottom panel: RNA polymerase from uninfected *M. smegmatis*.

( $\beta\beta'$  subunits do not separate well on 7.5% gels; see Bautz, 1976 and Wiggs *et al.*, 1979).

The molecular weights of the subunits were determined from the mobility of reference markers.

**Table 4.** Template specificity of RNA polymerase.

DNA template	RNA polymerase activity (nmol of [ <sup>3</sup> H]-UMP incorporated/5 min/mg protein)	
	Uninfected	Infected
Phage I3	13.96	48.83
<i>Mycobacterium smegmatis</i>	16.37	14.13
<i>Mycobacterium tuberculosis</i> H <sub>37</sub> R <sub>a</sub>	13.28	12.70
<i>Micrococcus lysodeikticus</i>	11.66	14.21
<i>Escherichia coli</i>	9.64	5.47
Phage $\lambda$	18.82	20.38
$\phi$ X174	7.82	6.96
Plasmid (pLM2)S <sub>4</sub>	18.28	17.94
Calf thymus	14.96	33.60
Salmon sperm	15.68	34.24
Poly d(A-T)	14.93	17.80
Poly (G-U)	4.61	3.36

Partially purified enzyme, after Ultrogel fractionation step was used. 10  $\mu$ g of protein and 5  $\mu$ g of different template DNAs (native) were used in each assay. RNA polymerase reaction was initiated by the addition of substrates. Details of assay are described in text.

coliphages T4 and T5 (Stevens, 1972; Doi, 1977; Szabo *et al.*, 1975). Similarly, infection with phages SP01 and SP82 results in altered promoter recognition and template specificity of RNA polymerase from *Bacillus subtilis* (Fox, 1976; Whitelay *et*

transcription by the phage SP01-modified (gene 28-product associated) RNA polymerase has been reported (Lee *et al.*, 1980). Coliphages T3 and T7 code for their own polymerases (Bautz, 1976; Doi, 1977) which carry out the transcription from late promoters. Another kind of mechanism is seen in coliphage N4 (Falco *et al.*, 1977, 1978) and phage PBS2 of *B. subtilis* (Clark *et al.*, 1974). These viruses have the RNA polymerase in the particles and is presumably injected into the host cell along with the DNA. The results presented in this paper clearly establish that the host RNA polymerase carries out phage I3 DNA transcription. Comparison of the structure of enzymes purified from uninfected and phage-infected cells show that the enzyme is in modified form after infection. A new polypeptide is found associated to the enzyme, in addition to the other subunits. The significant changes observed *in vitro* in the template specificity could be due to the binding of this factor to the host enzyme as no other changes were apparent in the enzyme structure.

The RNA polymerases from uninfected and phage I3-infected *M. smegmatis* bind to rifampicin-Sepharose column very tightly as seen from their elution profile. On the contrary, the RNA polymerase from *E. coli* is eluted at a much lower concentration of KCl indicating the lower affinity of this enzyme to rifampicin. This is consistent with the 10–100 fold higher sensitivity of mycobacterial enzymes to rifampicin, reported here and earlier for *M. tuberculosis* (Harshey and Ramakrishnan, 1976). However, mycobacterial enzyme seems to have less affinity towards heparin, because the enzyme is eluted from heparin-Sepharose column at a low salt (0.2 M KCl) concentration (data not shown). The procedure developed for purification of RNA polymerase is very rapid and the pure enzyme is obtained within 48 h. Although several procedures are available for the purification of RNA polymerases from prokaryotes, the method reported here using affinity chromatography on rifampicin-Sepharose columns could be adopted as a general method for the rapid purification of RNA polymerase from small amounts of starting material. This is the first report of the use of rifampicin-Sepharose as an affinity matrix for the purification of RNA polymerase.

Phage I3 DNA serves as the best template *in vitro* for the RNA polymerase from the phage-infected *M. smegmatis*. This immediately suggests the strong possibility that *in vivo*, the modified enzyme specifically transcribes phage I3 DNA, the bacterial DNA being less efficient. On the other hand, RNA polymerases from various sources show differential transcription of different templates or a particular template (Iida *et al.*, 1979; Wiggs *et al.*, 1979). However, maximum specificity is seen only with the phage-induced polymerases. For instance, enzymes from phages T3 and T7 selectively transcribe their own DNA to the maximum extent (Bautz, 1976). Another extreme example is coliphage N4 (Falco *et al.*, 1977, 1978). The purified N4 polymerase transcribes single stranded N4 DNA very efficiently but not native double stranded DNA. Phage I3-modified polymerase seems to prefer native DNA templates.

## Acknowledgement

This work was supported by the Department of Atomic Energy, New Delhi. (Project

## References

- Adhya, S., Gottesman, M., de Crombrugge, B. and Court, D. (1976) in *RNA polymerase* (eds R. Losick and M. Chamberlin), (New York: Cold Spring Harbor Laboratory) p. 719.
- Bautz, E. K. F. (1976) in *RNA polymerase* (eds R. Losick and M. Chamberlin), (New York: Cold Spring Harbor Laboratory) p. 273.
- Burgess, R. R. (1969) *J. Biol. Chem.*, **244**, 6160.
- Clark, S., Losick, R. and Pero, J. (1974) *Nature (London)*, **252**, 21.
- Doi, R. H. (1977) *Bacteriol. Rev.*, **41**, 568.
- Epp, C. and Pearson, M. L. (1976) in *RNA polymerase* (eds R. Losick and M. Chamberlin) (New York: Cold Spring Harbor Laboratory) p. 667.
- Fairbanks, G., Steck, C. L. and Wallach, D. F. H. (1971) *Biochemistry*, **10**, 2606.
- Falco, S. C., Laan, K. V. and Rothman-Denes, L. B. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 520.
- Falco, S. C., Zivin, R. and Rothman-Denes, L. B. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 3220.
- Fox, T. D. (1976) *Nature (London)*, **262**, 748.
- Franklin, N. C. and Yanofsky, C. (1976) in *RNA polymerase* (eds R. Losick and M. Chamberlin) (New York: Cold Spring Harbor Laboratory) p. 693.
- Gopinathan, K. P., Saroja, D., Gadagkar, R. R. and Ramakrishnan T. (1978) in *Genetics of Actinomycetales* (eds E. Freerksen, I. Tarnok and J. H. Thumin), (New York: Stuttgart, G. Fischer-Verlag) p. 237.
- Harshey, R. M. and Ramakrishnan, T. (1976) *Biochim. Biophys. Acta*, **432**, 49.
- Iida, H., Ikehara, K. and Okada, Y. (1979) *FEBS Lett.*, **99**, 346.
- Laemmli, U. K. (1970) *Nature (London)*, **227**, 680.
- Lee, G., Hannett, N. M., Karman, A. and Pero, J. (1980) *J. Mol. Biol.*, **139**, 407.
- Losick, R. and Chamberlin, M. (eds) (1976) in *RNA polymerase* (New York: Cold Spring Harbor Laboratory).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Nagaraja, V. and Gopinathan, K. P. (1980) *Arch. Microbiol.*, **124**, 249.
- Nagaraja, V. and Gopinathan, K. P. (1981) *Mol. Biol. Rep.*, **8**, 11.
- Smith, S. S. and Braun, R. (1978) *Eur. J. Biochem.*, **82**, 309.
- Stevens, A. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 603.
- Szabo, C., Dharmgrongartama, B. and Moyer, R. W. (1975) *Biochemistry*, **14**, 989.
- Travers, A. (1976) *Nature (London)*, **263**, 641.
- Whitelay, H. R., Spiegelman, G. B., Lawrie, J. M. and Hiatt, W. R. (1976) in *RNA polymerase* (eds R. Losick and M. Chamberlin) (New York: Cold Spring Harbor Laboratory) p. 58.
- Wiggs, J. L., Bush, J. W. and Chamberlin, M. J. (1979) *Cell* **16**, 97.



## Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein *via* conjugative plasmid F in *Escherichia coli* K-12

SHAMBHAVI SUBBARAO, NAM PRAKASH, A. V. SIVAPRASAD  
and SUSHIL KUMAR\*

Division of Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

**Abstract.** Expression of fimbriation was studied in *Escherichia coli* K-12 CA8000 HfrH, and its *cya*, *crp* and MS2 resistant mutants. The cells of *cya*<sup>+</sup> *crp*<sup>+</sup> parent strain were observed to be flagellated bacilli, lacking fimbriae, unable to agglutinate erythrocytes and deficient in ability to produce surface pellicle during growth in stationary culture. The cells of *cya* and *crp* mutants were observed to be cocci or coccobacilli devoid of flagella, having haemagglutinating activity, fimbriated and capable of producing surface pellicle in stationary cultures. The fimbriation and haemagglutinating activities were lower in *cya* mutants grown with cAMP supplementation. The *cya* and *crp* mutants produced relatively small, smooth and compact colonies consisting mostly of fimbriated cells, like those of earlier described Fim  $\sigma$  mutants. The *cya*<sup>+</sup> *crp*<sup>+</sup> MS2 resistant mutant produced large sized colonies like those of parent but was deficient in conjugal donor ability. It resembled *cya* and *crp* mutants in haemagglutinating and fimbriation properties. The *cya* and *crp* mutants have been earlier shown to be deficient in several Tra functions including conjugal donor ability. It is concluded that *Escherichia coli* K-12 cells express fimbriation when Tra functions of F-plasmid carried in them are not expressed either due to deficiency of active cAMP-receptor protein complex or mutation in F-plasmid or when F-plasmid is absent.

**Keywords.** *Escherichia coli* fimbriae; type 1 fimbriation; fimbriation under F-plasmid control; fimbriation under cyclic AMP control; cyclic AMP-receptor protein complex; conjugative F-plasmid.

## Introduction

Cells of certain Hfr, F<sup>+</sup> and F<sup>-</sup> sub-strains of *Escherichia coli* K-12 project from their surface short, rigid, hair like proteinaceous appendages called type 1 fimbriae (pili) (Brinton, 1959; Maccacaro and Hayes, 1961; Duguid and Old, 1980). The fimbriae are supramolecular hydrophobic structures about 7 nm in width consisting of helically arranged monomers of the 17 kd pilin polypeptide (Brinton, 1965). They are known to determine (i) the ability of cells to form pellicle on the surface of static broth cultures (Old and Duguid, 1970), and (ii) the adhesive properties for binding to a variety of erythrocytes and other eukaryotic cells (Duguid and Old, 1980; Parry and Rooke, 1985). Analogous structures on entero- and uro-pathogenic strains of *E. coli* have been shown to be involved in colonization leading to pathogenicity (Mooi and DeGraaf, 1985; Uhlin *et al.*, 1985). Stable *fim* (*pil*) mutants have been isolated in a F<sup>-</sup> substrain of *E. coli* K-12 and shown to map at 98 min on the conventional chromosome-map of *E. coli* K-12 (Swaney *et al.*, 1977a and b). Observations on the



phenotype of *fim* mutants have not provided any clue about the function of type 1 fimbriae of *E. coli* K-12.

Variation in fimbriation among some of the *E. coli* K-12 substrains has been studied and as a result two types of controls on fimbriation have been inferred.

(i) It has been observed, in certain sub-strains, that fimbriation is reversible. That is, fimbriated cells when grown on nutrient agar produce colonies composed largely of unfimbriated cells and contrariwise unfimbriated cells inoculated into broth and allowed to grow statically give rise to cultures in which fimbriated cells are preponderant. This kind of fimbriation phenotype has been designated as *Fim*<sup>+</sup> (Maccacaro and Hayes, 1961). The reversible fimbriation, also called as phase variation, has been attributed to a metastable regulatory element controlling transcription of fimbriae genes. In these bacteria the transcription switch for a cell oscillates between on and off states at a rate of  $10^{-3}$ /generation (Eisenstein, 1981). (ii) In certain other substrains of *E. coli* K-12, fimbriation has been reported to be relatively more profuse and more stable. This kind of phenotype has been designated as *Fim*  $\sigma$  (Brinton, 1959; Maccacaro and Hayes, 1961). The causal mechanism for *Fim*  $\sigma$  phenotype has not been outlined. In this connection, *Fim*  $\sigma$  like phenotype observed in the *E. coli* K-12 cells carrying strain J96 fimbriae genes has been associated with mutations in the *hyp* (hyperfimbriation) gene element of the *fim* gene-cluster (Orndoff and Falkow, 1984a and b).

The present study provides evidence that in *fim*<sup>+</sup> *E. coli* K-12 harboring F plasmid such as in the strain HfrH, the *cya* (adenyl cyclase deficient) and *crp* (cAMP receptor protein (CRP) deficient) mutants express stable fimbriation like that in previously described *Fim*  $\sigma$  mutants. It is also shown that fimbriation in *E. coli* K-12 *fim*<sup>+</sup> strains occurs only when the conjugative plasmid F is absent or expression from it is turned off.

## Material and methods

### *Strains and culture conditions*

The genotypes and phenotypes of the bacterial strains used are given in table 1. Bacterial cultures were grown in tryptone broth (TB), tryptone agar (TA) or minimal medium agar based on Davis-Mingioli salts. The growth temperature used was 30°C. The compositions of the media have been described (Kumar, 1976).

### *Electron microscopy*

Bacteria were grown as patches on TA medium. A loopful from a patch was suspended in 20  $\mu$ l of 0.01 M MgSO<sub>4</sub>. Suspension was placed on Formvar coated copper grid with a loop and allowed to dry. The grid was shadow casted with gold-palladium and lightly stained with phosphotungstic acid. Philips model 300 was used for the electronmicroscopy of the samples on grids.

### *Haemagglutination tests*

Table 1. Strains of *E. coli* K-12 used.

Strain	Genotype and derivation	Relevant phenotype	Reference(s)
CA8000	HfrH: thi, $\lambda^-$ , <i>refA1</i> , <i>min</i>	Wild type; rod shaped flagellated cells; minicell producer; unfimbriated; nonhaemagglutinating.	Brickman <i>et al.</i> (1973) and as given for the strain ED54
ED835	Spontaneous MS2 resistant mutant of CA8000	Phenotype of CA8000, but having transfer deficiency in conjugation; fimbriated; haemagglutinating	Present study
ED54	CA8000 harboring spontaneous <i>cya-4</i> point mutation	Multiple carbohydrate utilization deficiency; transfer deficiency in conjugation; resistance to $\lambda$ and T6 phages, ultraviolet light, $\gamma$ -rays, methyl-methanesulphonate, ampicillin, tetracycline, 1 M NaCl, hypotonic shock, heat shock at 50°C; sensitivity towards streptomycin, deoxycholate and sodium azide; cells shaped as cocci or coccobacilli; non-flagellated; inability to produce minicells; fimbriated; haemagglutinating	Kumar (1976), Kumar <i>et al.</i> (1979a,b, 1981), Kumar and Srivastava (1983), Gupta and Kumar (1985)
CA8306	CA8000 harboring <i>cya-854</i> deletion mutation	-do-	As given for the strain CA8000
ED56	CA8000 harboring spontaneous <i>cya-4</i> point mutation	-do-	As given for the strain ED54
ED836	CA8306 harboring <i>fin-48</i> mutation; isolated as nonhaemagglutinating mutant	Phenotype of CA8306 but non-fimbriated and non-haemagglutinating	Present study
ED837	CA8306 harboring <i>fin-64</i> mutation; isolated as nonhaemagglutinating mutant	Phenotype of CA8306 but fimbriated and nonhaemagglutinating	Present study

used. The red cells were separated by low speed centrifugation, washed suspended in 0.15 M NaCl and stored on ice. Bacteria to be tested were drawn from patches grown on agar medium. The media used were TA, nutrient agar, MacConkey agar and triple sugar iron agar (all Difco). To carry out a test about 20  $\mu$ l of erythrocyte suspension was taken on a microscope slide. A loopful of bacteria drawn from a patch were mixed into the suspension of blood cells. The slide was manually rotated a couple of times and examined after 3 min. Several tests were performed on the same slide and suitable controls were done on each slide. Diluted erythrocyte suspensions were used for quantifying haemagglutination.

### Other methods

Nitrosoguanidine (NG) was the mutagen employed. Stationary cultures were treated with about 50  $\mu$ g/ml NG for 4 h. After washing, cells were transferred to fresh broth and incubated with aeration for 12 h. The cultures were then diluted and plated on TA to recover discrete colonies. Other procedures used have been described previously (Kumar, 1976; Kumar and Srivastava, 1983).

## Results

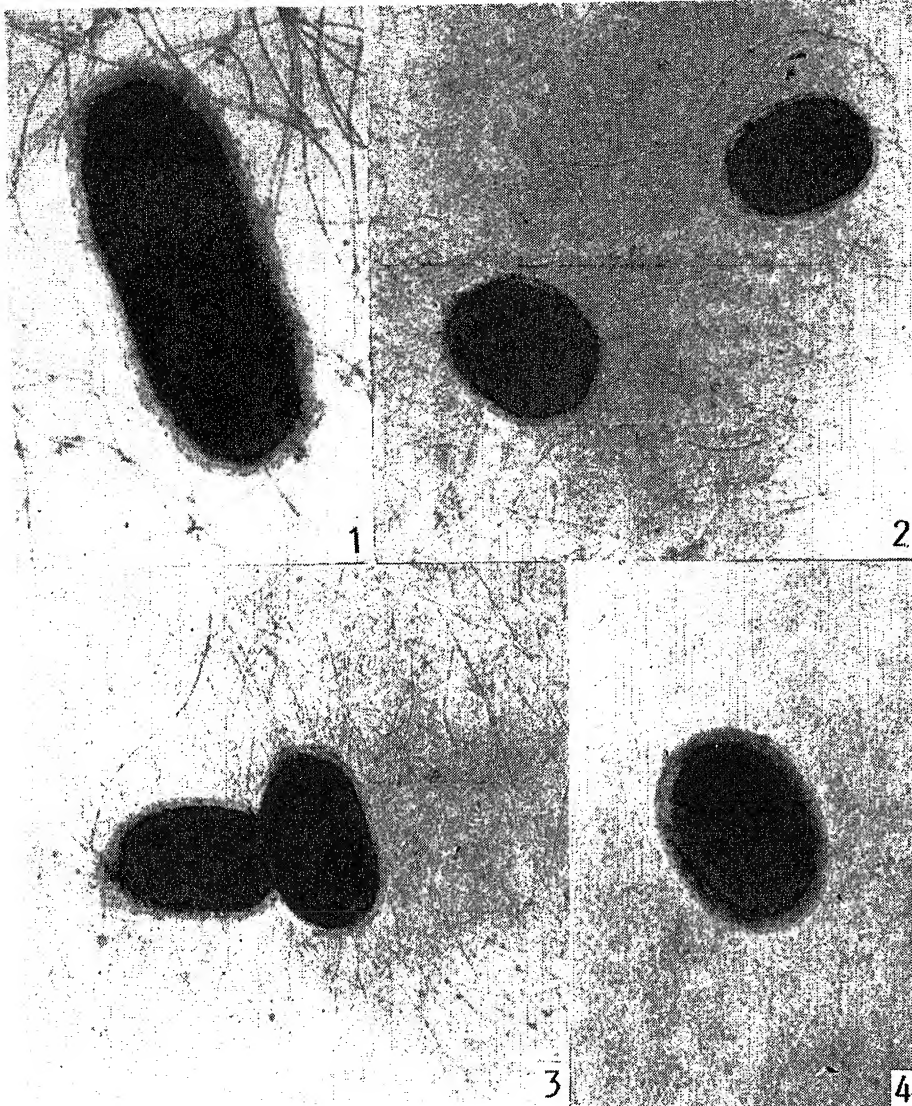
### *Fimbriation in the strain CA8000 and its cya and crp mutants*

Brinton (1959) and Maccacaro and Hayes (1961) examined several  $F^-$ ,  $F^+$  and Hfr strains of *E. coli* K-12 and observed that some of the strains of each kind were unfimbriated ( $Fim^-$ ). One of the Hfr strain they found unfimbriated was HfrH, isolated by Hayes (1953). The wild type strain CA8000 of the present study is a derivative of this HfrH strain. Strains ED54 (*cya*) and ED56 (*crp*) are one step spontaneous mutants derived from CA8000. Fimbriation in the 3 strains was compared (table 2 and figures 1 and 2). The cells of the *cya*<sup>+</sup> *crp*<sup>+</sup> HfrH strain CA8000 were found to be unfimbriated. On the other hand, cells of its *cya* and *crp* mutants were found to be fimbriated. It was also found that fimbriation on the cells of *cya* mutant was lower when they had been grown in the presence of  $5 \times 10^{-4}$  M cAMP as compared to

**Table 2.** Expression of fimbriation and flagellation by *E. coli* K-12 HfrH strain CA8000 and its *cya* and *crp* mutant derivatives.

Strain and medium-supplement if used	Total number of cells examined	Number of cells found		Mean number of appendages/cell	
		Fimbriated	Flagellated	Fimbriae	Flagella
CA8000	18	0	17	0	4.88
ED54 <sup>a</sup>	64	64	6	151	0.14
ED56	31	31	1	356	0.03
ED54 supplemented with $5 \times 10^{-4}$ M cAMP	21	8	11	38	2.05

<sup>a</sup>CA8306 cells were found to be poorly flagellated but densely fimbriated like those of

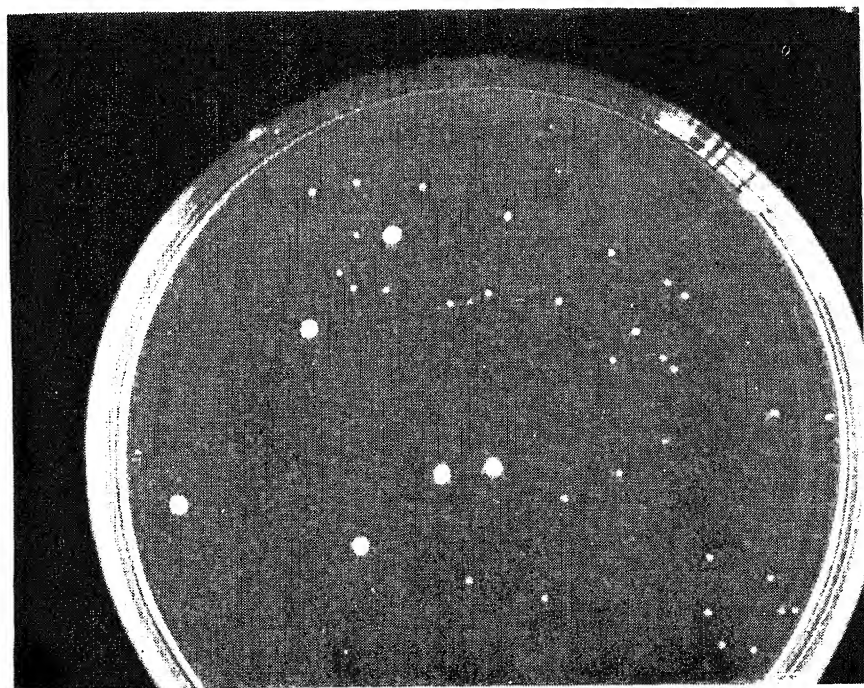


**Figures 1-4.** Electronmicrographs of *E. coli* K-12 CA8000 HfrH and its derivatives. 1. A cell of the strain CA8000, rod shaped, flagellated and lacking fimbriae. A fraction of ED54 cells grown with cAMP supplementation looked like this cell. 2. Two cells of the strain ED54 (*cya*); shaped as cocco-bacilli, fimbriated but without flagella. A broken flagellum can be seen in the background. The cells of strains ED56 and CA8306 looked like these. 3. Two cells of the strain ED837 (*cya* and haemagglutinating mutation)-surface features are identical to ED54 cells. 4. A cell of the strain ED836 (*cya*, *fim*), shaped as cocco-bacillus and lacking both flagella and fimbriae.

when grown in the absence of cAMP. Contrariwise, the cells of *crp* mutant grown in the presence or absence of cAMP were found to be about equally fimbriated (data not included). These results were taken to suggest that fimbriation in the *E. coli* K-12

In their work Macassar and Hayes (1961) designated a class of slowly fimbriated mutants of *E. coli* K-12 as *Fim*  $\sigma$ . They observed that *Fim*  $\sigma$  mutants produced small, smooth and compact colonies on nutrient agar. The colonies were entirely composed of fimbriated cells. Recently, Orndoff and Falkow (1984b) reported stably fimbriated mutants in *E. coli* strains harboring fimbriae genes of the heterologous strain J96. These hyperfimbriated (*hyp*) mutants produced colonies similar to those described for *Fim*  $\sigma$  mutants.

Previous work (Kumar, 1976) has established that the *cya* and *crp* mutants of *E. coli* K-12 CA8000 produce small and smooth colonies relative to larger and roughish colonies of the parent strain. Measurements of the diameters of the 48 h old colonies produced by 3 strains on tryptone agar revealed that the average area of the CA8000 colonies is about 5 fold larger than that of both *cya* and *crp* mutants (figure 5). The number of colony forming units/colony for the strain CA8000 was established to be about  $2 \times 10^6$ . The corresponding figure for *cya* and *crp* mutant colonies was about  $10^6$ . These data indicated that colonies of *cya* and *crp* mutants of CA8000 must be relatively compact. Since the colonies of *cya* and *crp* mutants proved to be smoother, smaller and more compact than the colonies of the *cya*<sup>+</sup> *crp*<sup>+</sup> parent strain, it is suggested that *cya* and *crp* mutants possess *Fim*  $\sigma$  phenotype.



**Figure 5.** Colonies of CA8000 and ED54 strains of *E. coli* K-12. Large colonies are of CA8000 and smaller ones of ED54. The colonies of the strain ED835 look like those of CA8000 and those of ED56, CA8306, ED836 and ED837 like those of ED54.

*Surface-pellicle formation in the stationary cultures of cya and crp mutants*

Each of the 72–96 h stationary cultures of ED54 and ED56 strains grown in tryptone broth had surface pellicle. In the culture tubes, the pellicle was visualized as a layer of bacteria on the surface of liquid medium or thick layer of growth stuck to the culture tube wall just about the surface level. Pellicle formation was not observed in the cultures of CA8000. Thus correlation was observed between fimbriation and pellicle formation.

*Haemagglutination properties of cya and crp mutants*

Haemagglutination tests were performed on the strains CA8000, CA8306, ED54 and ED56 grown on a variety of solid agar media. Both rabbit and chicken erythrocytes were employed. Complete correspondence was noted between fimbriation and haemagglutinating properties of these strains (table 3). Haemagglutination was not seen with CA8000 cells. The cells of CA8306, ED54 and ED56 caused strong haemagglutination. When cells of CA8306 and ED54 grown on medium supplemented with  $10^{-3}$  M cAMP or 0.5 M NaCl were used, weaker haemagglutination was observed.

**Table 3.** Haemagglutination properties of *E. coli* K-12 strain CA8000 HfrH and its *cya* and *crp* mutant derivatives.

Strain	Agglutination <sup>a</sup> of erythrocytes of	
	Rabbit	Chicken
CA8000	—	—
ED54 <sup>b</sup>	+	+
ED56	+	+
CA8306	+	+

<sup>a</sup>Cells for these tests were taken from patches of growth produced on tryptone agar, nutrient agar or triple sugar iron agar, all Difco.

<sup>b</sup>Haemagglutination with ED54 was found to be mannose sensitive.

*Fimbriation on non-haemagglutinating mutants of ED54*

The strong haemagglutinating property of *cya* strains was used to isolate nonhaemagglutinating mutants. Cultures of the strains CA8306 and ED54 were mutagenized with nitrosoguanidine and plated on TA to obtain discrete colonies. These colonies were purified by streaking and then tested for inability to utilize lactose, maltose and arabinose and for resistance to phage  $\lambda$  to confirm the presence of original *cya*

ages. Among the mutants, two classes were detected; a class of mutants were unfimbriated (figure 4) and mutants of the other class were fimbriated (figure 3). These observations suggested that in *E. coli* K-12, the haemagglutinating function is complex and fimbriae can be of two types—haemagglutinating and nonhaemagglutinating. Whereas haemagglutination is a test for the presence of fimbriae, nonhaemagglutination does not indicate absence of fimbriae.

### *Haemagglutination and fimbriation properties of MS2 resistant mutants of CA8000*

Earlier it has been shown that *cya* and *crp* mutants of the strain CA8000 fail to produce sex pili on account of poor expression of Tra functions in the absence of active cAMP-CRP complex in these mutants (Kumar and Srivastava, 1983). This finding and the present observation that *cya* and *crp* mutants of the strain CA8000 express well their fimbriation functions led us to the question whether there is any direct relationship between the expression from integrated F factor and lack of fimbriation in the wild type HfrH. To examine this question, strain ED835 was isolated as a spontaneous tightly MS2 resistant mutant of CA8000 and characterized. ED835 was found to be strongly haemagglutinating and fimbriated like ED54 and ED56. Thus the results suggested that lack of fimbriation must be correlated with expression of Tra functions from the F-factor located on the HfrH chromosome.

### **Discussion**

In the experiments reported here, one of the observations is that in *E. coli* K-12 CA8000 HfrH strain, the wild type *cya*<sup>+</sup> *crp*<sup>+</sup> bacteria are nonhaemagglutinating, unable to form pellicle during growth in static broth cultures and unfimbriated and isogenic *cya* and *crp* mutants are haemagglutinating, pellicle forming and fimbriated. These results mean that cAMP (deficient in *cya* mutants) and its receptor protein (deficient in *crp* mutants) negatively regulate expression of *fim* genes. In relation to this conclusion it is necessary to reinterpret here the divergent observations of Eisenstein *et al.* (1981) and Eisenstein and Dodd (1982) on fimbriation in *cya*<sup>+</sup> and *cya* strains of *E. coli* K-12. Eisenstein and coworkers, in their study reported in 1981, had observed *E. coli* K-12 CA8000 HfrH cultures to be poorly fimbriated. They had observed cultures of its *cya* mutant CA8306 as fimbriated. Decrease in fimbriation on addition of cAMP to CA8306 cultures was also observed by them. However, their observations on the strain 5336 (Perlman and Pastan, 1969) diverted them from implicating cAMP in the negative control of fimbriation. The cells of strain 5336 were found to possess few fimbriae and fimbriation on them improved by supplementation with cAMP, the latter, like in a strain of *Salmonella typhimurium* (Saier *et al.*, 1978). In their study strain 1101, the immediate parent of 5336, from which 5336 had been derived by mutagenization with NG had not been included. The strain 1101 itself had been earlier derived from strain 3000 after treatment with NG (Fox *et al.*, 1967; Fox and Wilson, 1968). Fimbriation properties of strains 3000 and 1101 are not known. It will be of interest to note that a sister strain of 1101 called 1100 improved  $\beta$ -galactosidase expression 2-fold when cAMP was supplied to it exogenously (Perlman and Pastan, 1969). Thus observed differences between the fimbriation

phenotypes of *cya* mutants CA8000 and 5550 reported by Eisenstein *et al.* (1981) could be attributed to inherent differences in their genetic backgrounds. Interestingly, Eisenstein and Dodd (1982) next studied effect of *cya* mutation on fimbriation in the CSH50 F<sup>-</sup> strain of *E. coli* K-12. In this study the isogenic strains CSH50 (*cya*<sup>+</sup>) and VL391 (*cya*-854) were both found to be fimbriated. Fimbriation of VL391 was not reduced much by the presence of cAMP in the medium. Eisenstein and Dodd (1982) did not note the negative effect of cAMP on fimbriation because, as shown in the present study, the effects of cAMP and its receptor protein on fimbriation are mediated *via* expression of Tra genes from the conjugative plasmid F.

Second observation relates to the Fim  $\sigma$  (Macaccaro and Hayes, 1961) phenotype of *cya* and *crp* mutants. It has been observed in this study that *cya* and *crp* mutants of *E. coli* K-12 CA8000 HfrH are stably fimbriated and produce small, smooth and compact colonies on Bacto-tryptone based agar medium.

In the present study it has also been observed that MS2 resistant mutant of *E. coli* K-12 CA8000 HfrH is fimbriated. Considering that *cya* and *crp* mutants in the same genetic background are also MS2 resistant and fimbriated, it is suggested that negative control of cAMP and its receptor protein on fimbriation is indirect. The cAMP-CRP complex is required for the expression of transfer genes of conjugative plasmid F and one or more *tra* gene products negatively regulate expression of *fim* operon(s). The conclusion reached here explains the observations on *cya*<sup>+</sup> and *cya* versions of CSH50 discussed above. Additional support to the above conclusion comes from the phenotypes reported by McEwen and Silverman (1980, 1982) for *E. coli* K-12 AE1031 Hfr strain and its derivatives carrying temperature sensitive mutations *cpx-B1* and *cpx-A2*. The cells of *cpx-A cpx-B* double mutant grown under nonpermissive conditions were characterized as round, fimbriated, lacking sex pili and deficient in conjugational DNA transfer. In addition, their cell walls were noted to carry rather large amounts of protein(s) in the range of 17kd molecular weight. Contrariwise, *cpx-A*<sup>+</sup> *cpx-B*<sup>+</sup> cells and those of *cpx-A*<sup>+</sup> *cpx-B1* grown under permissive conditions were found to be rods having sex pili but poorly fimbriated. Their cell walls were found to carry very small amounts of 17kd protein. Thus when Hfr function was inactivated by expression of *cpx-A* and *cpx-B* mutations, *E. coli* K-12 AE1013 appeared fimbriated. It is possible that *cpx-A* and *cpx-B* function in concert with cAMP-CRP complex or that expression of *cya*, *crp* or both genes is under the control of *cpx-A* and *cpx-B*. Besides *cya*, *crp*, *cpx-A* and *cpx-B*, *sfr-A*, *sfr-B* (Beutin and Achtman, 1979), *fex-A* and *fex-B* (Lerner and Zinder, 1982) are also known to be required for expression of Tra functions from F-plasmid. But the phenotypes of mutants in the latter genes, in relation to fimbriation, are not known.

It appears that interaction within and between 3 sets of genes determine fimbriation properties of *E. coli* K-12. These 3 groups of genes are: (i) various chromosome-borne genes, including *cya*, *crp*, *cpx-A*, *cpx-B*, *fex-A*, *fex-B*, *sfr-A*, *sfr-B*, and *ilv-B*; (ii) genes present on the genome of F-plasmid; (iii) regulatory, structural and assembly genes specific for fimbriation. The details of roles of these genes in the process of fimbriation remain to be elucidated.

At the end a few related questions about the importance of fimbriation in the life of bacteria are perhaps pertinent. Two of these are: (i) Why expression of F-plasmid mediated DNA donorability requires the absence of fimbriation? (ii) Why fimbriation can occur constitutively in F<sup>-</sup> bacteria? Fimbriated DNA-donor



between F<sup>-</sup> and unfimbriated male bacteria. There may be specific receptors free for pilin binding on the surface of unfimbriated male bacteria but occupied on fimbriated bacteria. Third question is: Why is there phase variation in fimbriation? Phase variation permits development of populations of fimbriated as well as unfimbriated cells in a culture. At any given location for bacteria there are likely to be micro-environments, naturally present or created by growth of bacteria. Some of these may be more suitable for fimbriated bacteria and others for unfimbriated ones. For example in stationary cultures, oxygen rich surface is more suitable for fimbriated bacteria. Fimbriated bacteria can take advantage of surface on account of hydrophobic nature of their pili (Brinton, 1965; Salit and Gotschlich, 1977). Thus control of fimbriation by phase variation in bacteria may be means for their exploiting the various microenvironments of a milieu.

## Acknowledgements

Grateful thanks are due to Dr. Pushpa Aggarwal for providing chicken and rabbit blood samples from laboratory reared animals and to Drs. T. V. Subbaiah and S. Ramachandran for helpful discussions on the subject of fimbriae.

## References

- Beutin, L. and Achtman, M. (1979) *J. Bacteriol.*, **139**, 730.  
 Brickman, E., Soll, L. and Beckwith, J. (1973) *J. Bacteriol.*, **116**, 582.  
 Brinton, C. C. Jr. (1959) *Nature (London)*, **183**, 782.  
 Brinton, C. C. Jr. (1965) *Trans. N. Y. Acad. Sci.*, **27**, 1003.  
 Duguid, J. P. and Old, D. C. (1980) in *Bacterial adherence* (ed. E. H. Beachey) (London: Chapman and Hall) p. 185.  
 Eisenstein, B. I. (1981) *Science*, **214**, 337.  
 Eisenstein, B. I. and Dodd, D. C. (1982) *J. Bacteriol.*, **151**, 1560.  
 Eisenstein, B. I., Beachey, E. H. and Solomon, S. S. (1981) *J. Bacteriol.*, **145**, 620.  
 Fox, C. F., Carter, J. R. and Kennedy, E. P. (1967) *Proc. Natl. Acad. Sci. USA*, **57**, 698.  
 Fox, C. F. and Wilson, G. (1968) *Proc. Natl. Acad. Sci. USA*, **59**, 988.  
 Gupta, A. and Kumar, S. (1985) *Indian J. Exp. Biol.*, **23**, 647.  
 Hayes, W. (1953) *Cold Spring Harb. Symp. Quant. Biol.*, **18**, 75.  
 Kumar, S. (1976) *J. Bacteriol.*, **125**, 545.  
 Kumar, S., Agarwal, K. N. and Hazela, S. (1981) *Indian J. Exp. Biol.*, **19**, 640.  
 Kumar, S., Prakash, N. and Agarwal, K. N. (1979a) *Indian J. Exp. Biol.*, **17**, 325.  
 Kumar, S., Prakash, N. and Sharma, V. K. (1979b) *Mol. Gen. Genet.*, **176**, 449.  
 Kumar, S. and Srivastava, S. (1983) *Mol. Gen. Genet.*, **190**, 27.  
 Lerner, T. J. and Zinder, N. D. (1982) *J. Bacteriol.*, **150**, 156.  
 Maccacaro, G. A. and Hayes, W. (1961) *Genet. Res.*, **2**, 394.  
 McEwen, J. and Silverman, P. (1980) *J. Bacteriol.*, **144**, 60.  
 McEwen, J. and Silverman, P. M. (1982) *J. Bacteriol.*, **151**, 1553.  
 Mooi, F. R. and DeGraaf, F. K. (1985) *Curr. Top. Microbiol. Immunol.*, **118**, 119.  
 Old, D. C. and Duguid, J. B. (1970) *J. Bacteriol.*, **103**, 447.  
 Orndoff, P. E. and Falkow, S. (1984a) *J. Bacteriol.*, **159**, 736.  
 Orndoff, P. E. and Falkow, S. (1984b) *J. Bacteriol.*, **160**, 61.  
 Parry, S. H. and Rooke, D. M. (1985) in *The virulence of Escherichia coli* (ed. M. Sussman) (Academic Press) p. 79.  
 Perlman, R. L. and Pastan, I. (1969) *Biochem. Biophys. Res. Commun.*, **37**, 151.  
 Saier, M. H. Jr., Schmidt, M. P. and Liebowitz, M. (1978) *J. Bacteriol.*, **132**, 328.

Salit, I. E. and Gotschlich, E. C. (1977) *J. Exp. Med.*, **146**, 1169.

Swaney, L. M., Liu, Y.-P., To, C.-M., To, C.-C., Ippen-Ihler, K. and Brinton, C. C. Jr. (1977a) *J. Bacteriol.*, **130**, 495.

Swaney, L. M., Liu, Y.-P., Ippen-Ihler, K. and Brinton, C. C. Jr. (1977b) *J. Bacteriol.*, **130**, 506.

Uhlen, B. E., Baga, M., Goronsson, M., Lindberg, F. P., Lund, B., Norgren, M. and Normark, S. (1985) *Curr. Top. Microbiol. Immunol.*, **118**, 163.



# Stabilisation of some of the protein synthesis components in the thermophilic fungus, *Humicola lanuginosa*

ANIL K. JOSHI\* and JOSEPH D. CHERAYIL

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

\*Present address: Department of Biochemistry, University of California, Berkeley, California 94720, USA

**Abstract.** The thermal stabilities of tRNA from the thermophilic fungus, *Humicola lanuginosa* were compared with that from the mesophilic yeast, *Candida utilis*, by measuring the increase in the optical density with temperature. tRNAs from both the species were stable in the presence of millimolar quantities of magnesium chloride upto 50°C, the optimum growth temperature of the fungus. Aminoacyl tRNA synthetases were maximally active at 40°C under the *in vitro* assay conditions. They were fractionated and one species of valine tRNA synthetase was purified to homogeneity. The purified enzyme was protected against inactivation to varying degrees when preincubated with the substrates valine, tRNA and ATP as well as spermine. Protein turnover studies showed that the rate of turnover was higher at higher temperatures. It was concluded from these results that the protein synthesizing machinery of this fungus has no intrinsic stability but it is stabilised by intracellular factors. Higher rate of protein turnover also plays a role for growth at higher temperature.

**Keywords.** Thermophilic fungus; stabilisation of synthetase; rate of protein turnover; *Humicola lanuginosa*.

## Introduction

A variety of microorganisms have the ability to grow optimally at temperatures much higher than the ordinary and some of them can grow well at the boiling point of water. But no eukaryote has been reported to grow at temperatures beyond 60°C. As both prokaryotes and eukaryotes comprise of aerobic organisms with simple nutritional requirements the inability of eukaryotes, especially fungi, to grow at temperatures beyond 60°C is surprising. Attempts have been made to understand the molecular basis of the upper temperature limit for the growth of eukaryotes. Tansey and Brock (1978) have suggested that thermophilic eukaryotes are unable to synthesise intracellular membranes that are functional beyond 60°C. However, experimental proof for such a postulate is lacking. Studies on the lipids of thermophilic species have shown that these species produce lipids with a high content of saturated fatty acids (Sumner and Morgan, 1969; Sumner *et al.*, 1969). These results suggest that the types of lipids the organism is able to produce determine the temperature of its growth.

The biological activity of proteins is of prime importance for growth at the optimum temperature of an organism. A large number of proteins from thermophilic bacteria, especially *Bacillus stearothermophilus* and *Thermus aquaticus* have been purified and characterised (Ljungdahl and Sherod, 1976; Amelunxen and Murdock, 1978). Some of these proteins have been found to be very stable while others not so stable. They have been classified into 4 groups on the basis of their thermostability *in vitro*. The proteins which are labile are postulated to be either stabilised by the

Amelunxen, 1975). The majority of proteins from thermophilic bacteria, however, are intrinsically stabler than the corresponding proteins from mesophilic organisms. Only a small number of proteins from thermophilic fungi have been studied. There is, in general, a lack of information on thermophilic eukaryotes and fungi, in particular. The available data, however, show that most intracellular proteins from thermophilic fungi, unlike that from thermophilic bacteria, are thermolabile *in vitro* around the growth temperature of the organism (Prasad and Maheshwari, 1978a; Wali *et al.*, 1979). In contrast, many extracellular proteins from these organisms are thermostable (Loginova and Tashpulatov, 1967; Rao *et al.*, 1979, 1981). From these studies it appears that a large proportion of proteins from thermophilic fungi do not exhibit the thermostability required to grow at their optimum growth temperatures. It is interesting to know how these organisms carry out protein synthesis at their optimum temperatures of growth. The thermal characteristics of the protein synthetic machinery are of interest in this connection. We present here the results of our studies on the thermostability of tRNA, and aminoacyl-tRNA synthetases as well as protein turnover in *Humicola lanuginosa*, a commonly found thermophilic fungus which has an optimum temperature of growth at 50°C and upper temperature for growth at 60°C.

## Materials and methods

*Humicola lanuginosa* was kindly provided by Dr. R. Maheshwari of this Department and the mesophilic yeast *Candida tropicalis* was from the Microbiology and Cell Biology Laboratory of this Institute. [ $^{14}\text{C}$ ]-Algal protein hydrolysate (24 mCi/matom) and [ $^{14}\text{C}$ ]-valine (30 mCi/mmol) were from Bhabha Atomic Research Centre, Bombay. [ $^{14}\text{C}$ ]-L-Leucine (339 mCi/mmol) and [ $^3\text{H}$ ]-L-leucine (18 Ci/mmol) were from Radiochemical Centre, Amersham, England. The ion-exchange materials and other fine chemicals were from Sigma Chemical Co., St. Louis, Missouri, USA. All other chemicals were of analytical grade.

### Isolation of tRNA

*H. lanuginosa* cells were grown in a synthetic medium (Prasad and Maheshwari, 1978b) with L-asparagine concentration raised to 0.4% in a temperature controlled rotary shaker at 50°C. After 10–12 h the cells were harvested by filtering and tRNA was isolated by extraction with phenol (Von Ehrenstein, 1967). Contaminating polysaccharides were removed by selective precipitation with cetyltrimethylammoniumbromide (Bellamy and Ralph, 1968) and the polysaccharide-free tRNA was deacylated according to the method of Harris (1978). *C. utilis* was grown in an enriched medium at 30°C, the cells were collected by centrifugation and the tRNA was isolated in a similar manner.

### Thermostability measurements

Thermal denaturation of tRNA was monitored by measuring the hyperchromicity at 260 nm with increase in temperature in 10 mM cacodylate buffer, pH 7.2 containing

5 mM NaCl and specified amounts of  $\text{MgCl}_2$  or spermine. About one absorbance (260 nm) unit/ml of tRNA preparation in a final volume of 250  $\mu\text{l}$  was used to monitor the increase in absorbance at 2°C increments in a Beckman DU-8B recording spectrophotometer.

### *Pancreatic RNase digestion*

Susceptibility of tRNA to pancreatic RNase was monitored by measuring the increase in absorbance at 260 nm with time as indicated above. A known quantity of RNase A was mixed with 500  $\mu\text{l}$  of the tRNA preparation (about one absorbance (260 nm) unit/ml) in 10 mM cacodylate buffer, pH 7.2 containing 60 mM KCl and 10 mM  $\text{MgCl}_2$  and the increase in absorbance (260 nm) was monitored at a specified temperature as a function of time at one minute intervals.

### *Rate of protein turnover*

The method used to study the rate of protein turnover was an adaptation of the double isotope method of Arias *et al.* (1969). A 20 ml medium was inoculated with freshly grown *H. lanuginosa* cells and grown for 6 h at 50°C in a rotary shaker. The cells were shifted to the specified temperature and after 5 min 5  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]-L-leucine was added. After labelling for 30 min the cells were harvested by mild suction through millipore membrane and washed well with the growth medium. The cells were then suspended in 20 ml of fresh medium and allowed to grow for 2 h at the same temperature. This was to allow turnover of the [ $^{14}\text{C}$ ]-label already incorporated into the protein. The cells were collected and an amount equal to that at the end of the [ $^{14}\text{C}$ ]-pulse period was grown for 10 min with 25  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-L-leucine. The remaining cells were allowed to grow in the non-radioactive medium at the same temperature for 10 min. The two types of cells were mixed, filtered and washed thoroughly with ice-cold medium. The washed cells were ground with sand and the lysate was extracted with 5 ml of a buffer containing 50 mM Tris-HCl, pH 7.2 and 25 mM NaCl. The extract was centrifuged at 8,000 *g* for 10 min and an aliquot of the supernatant fraction was spotted on Whatman 3 mm paper and thoroughly washed with 10% hot trichloroacetic acid (TCA) containing 0.1% L-leucine and ethanol-ether mixture. The paper was dried and counted for [ $^{14}\text{C}$ ]- and [ $^3\text{H}$ ]-labels in a liquid scintillation counter. The less the number of [ $^{14}\text{C}$ ]-counts at the end, the more will be the protein turnover. Thus the ratio [ $^3\text{H}$ ]/[ $^{14}\text{C}$ ]-is a measure of the rate of protein turnover.

### *Aminoacyl tRNA synthetase assay*

*H. lanuginosa* cells (2–5 g), grown for 8–10 h were ground with an equal amount of sand and extracted with chilled buffer containing 10 mM Tris-HCl, pH 7.2, 10 mM  $\text{MgCl}_2$ , 30 mM  $\text{NH}_4\text{Cl}$ , 5 mM  $\beta$ -mercaptoethanol and 10% glycerol. The cell lysate was centrifuged at low speed and the supernatant fraction was further centrifuged at

The standard assay mixture contained 50 mM Tris-HCl, pH 7.2, 2 mM ATP, 0.5 mM CTP, 10 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, 0.5 mM EDTA, 0.1  $\mu$ Ci [<sup>14</sup>C]-algal protein hydrolysate (24 mCi/m atom) about 4 absorbance (260 nm) units of tRNA and about 10  $\mu$ g of enzyme protein in a total volume of 200  $\mu$ l. After incubation at 37°C for a specified period 150  $\mu$ l of the reaction mixture was applied to a Whatman 3 mm paper, presoaked in 10% TCA and was washed thoroughly with TCA solution containing 0.1% casamino acids. The paper was dried and counted.

Valine tRNA synthetase assay was carried out in a similar manner except that the composition of the assay mixture was different. It contained 50 mM Tris-HCl, pH 8.0, 10 mM KCl, 3 mM ATP, 15 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, 0.5 mM EDTA, 180  $\mu$ M [<sup>14</sup>C]-L-valine (30 mCi/mmol) about 50 absorbance (260 nm) units of tRNA and about 170 ng of purified valine tRNA synthetase in a final volume of 200  $\mu$ l.

## Results

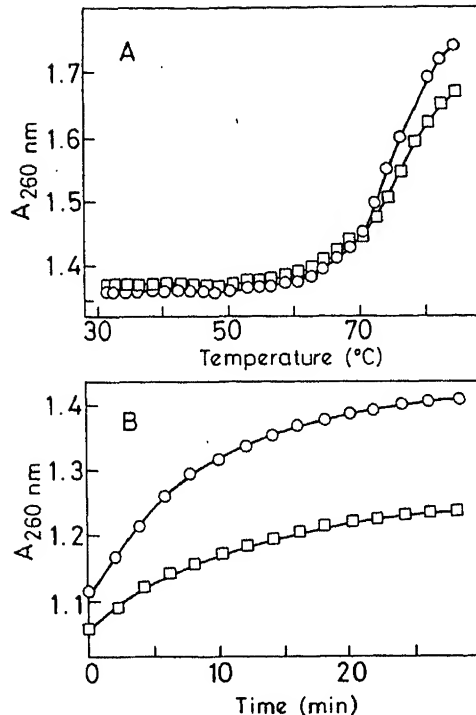
### *The stability of tRNA*

The stability of the tRNAs is an important factor which determines the upper temperature of growth of an organism. Hence the thermal melting profiles of total tRNA from *H. lanuginosa* were compared with that of mesophilic yeast, *C. utilis*. Increase in the optical density was measured against temperature in the buffer with different concentrations of MgCl<sub>2</sub> or spermine. tRNA from both sources had almost identical melting profiles suggesting similar stability. Although *H. lanuginosa* tRNA melted at temperatures lower than the optimum temperature of its growth in the absence of any added MgCl<sub>2</sub> or spermine, addition of small quantities of these raised the temperature of melting. In 2.5 mM MgCl<sub>2</sub> the temperature at which *H. lanuginosa* tRNA started melting was 56°C and the midpoint of melting was 76°C. The tRNA was thus stable at the optimum temperature of its growth, but did not have any special feature different from that of *C. utilis* tRNA which was also stable up to about 56°C (figure 1A).

Rate of digestion of a nucleic acid with a nuclease roughly indicates the degree of compactness of its structure. The tRNA samples were digested with RNase A at different temperatures to get an insight into their tertiary structure. *H. lanuginosa* tRNA was digested at a slower rate by the enzyme (figure 1B).

### *Stability of aminoacyl tRNA synthetases*

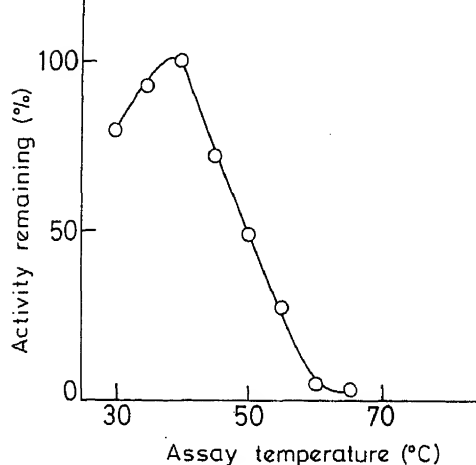
Studies were carried out with the total synthetase preparation, freed of the endogenous tRNA and amino acids by batch-wise elution from DEAE-Sephacel, to establish the optimum conditions for assay. Thus the pH, the concentration of ATP, magnesium ions and other components as well as the time of incubation for linear response were determined using <sup>14</sup>C-algal protein hydrolysate (data not presented). The effect of temperature on the aminoacylation reaction was studied by carrying out the reaction at various temperatures. The acceptance increased with temperature



**Figure 1.** Melting profile of tRNA and the rate of digestion with RNase. **A.** Melting profile of tRNA: a solution of tRNA in 10 mM buffer, pH 7.2, containing 5 mM NaCl and 2.5 mM MgCl<sub>2</sub> was taken in a 250  $\mu$ l cuvette and absorbance against temperature was recorded in a DU-8B Beckman Recording Spectrophotometer at 2 min intervals. **B.** Rate of digestion with pancreatic RNase: 2.5  $\mu$ g of RNase was mixed with 500  $\mu$ l of tRNA preparation in 10 mM cacodylate buffer, pH 7.2, containing 60 mM KCl and 10 mM MgCl<sub>2</sub> at 37°C. Increase in absorbance was monitored as a function of time at 1 min intervals. ( $\square$ ), *H. lanuginosa* tRNA; ( $\circ$ ), *C. utilis* tRNA.

from 30°C to 40°C and then decreased sharply with rise in temperature (figure 2). The activity at 50°C, the temperature for optimum growth of the organism, was only about half of that at 40°C and at 60°C, the maximum growth temperature was very low. These results suggested that in the *in vitro* reaction all the factors that stabilise the enzymes were not present. It was also not clear whether the *in vitro* lower activity at higher temperatures was due to reversible thermal inactivation or due to irreversible thermal denaturation of the enzymes. Only by the use of a homogeneous single enzyme this could be clarified. The synthetase preparation was therefore fractionated by ammonium sulphate precipitation, chromatography on CM-50 Sephadex (von der Haar, 1979) interfacial salting out on a Sepharose-4B column by a reverse gradient (von der Haar, 1976) and affinity chromatography. Upon chromatography on the CM-50 Sephadex column valine tRNA synthetase resolved into two peaks, a minor peak (valine tRNA synthetase<sub>1</sub>) and a major peak (valine tRNA synthetase<sub>2</sub>). On further fractionation valine tRNA synthetase<sub>2</sub> gave a single protein as shown by electrophoresis on polyacrylamide gels under native and denaturing conditions. This





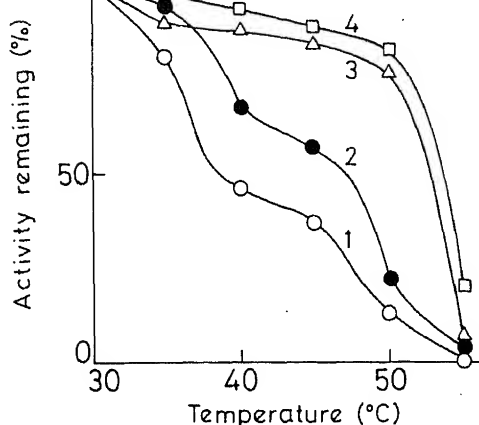
**Figure 2.** Effect of temperature on aminoacyl tRNA synthetase activity.

The aminoacylation assay for total synthetase was carried out at different temperatures using [ $^{14}\text{C}$ ]-algal protein hydrolysate. The maximum activity obtained was taken as 100% to plot the values at different temperatures.

however, be purified to a homogeneous state by this procedure. Details of the purification of valine tRNA synthetase<sub>2</sub> will be published elsewhere.

#### *Studies with valine tRNA synthetase<sub>2</sub>*

Experiments were again carried out to establish the optimum conditions for assay with total tRNA using [ $^{14}\text{C}$ ]-L-valine for label (data not presented). Study of the effect of temperature on the aminoacylation reaction showed that as in the case of the total tRNA synthetase preparation the maximum activity of valine tRNA synthetase<sub>2</sub> was below the optimum growth temperature of the organism (see figure 2). However, the maximum activity was at 45°C, slightly higher than that for the total tRNA synthetase. At 50°C the activity was nearly 85% of that at 45°C but at 60°C the activity was only about 10% of that at 45°C. In order to distinguish whether the low activity at higher temperatures was due to heat inactivation or irreversible thermal denaturation of the protein, the following experiments were carried out. The enzyme was heated at different temperatures in the presence of various effector agents for 5 min, cooled and assayed at 40°C. In the absence of the substrates at the time of preincubation the enzyme was highly thermolabile. It lost more than 50% activity by heating for 5 min at 40°C and more than 90% by heating at 50°C. It was clear that the enzyme was irreversibly inactivated. The substrates, L-valine, tRNA and ATP were able to protect the enzyme to some extent against heat denaturation individually and in combination. Among the substrates tRNA was the most effective. These substrates in combination could give a great deal of protection to the enzyme (figure 3). In addition, spermine in very small quantities could also protect the enzyme to some extent (data not presented).



**Figure 3.** Protection of valine tRNA synthetase<sub>2</sub> by substrates at different temperatures.

Valine tRNA synthetase<sub>2</sub> in 50 mM Tris-HCl buffer, pH 8.0 containing 10 mM KCl, 5 mM  $\beta$ -mercaptoethanol and definite quantity of the effector was heated at various temperatures for 5 min, cooled in ice and assayed at 40°C.

(1), No addition; (2), 180  $\mu$ M L-Valine; (3), 50 absorbance (260 nm) units of tRNA; (4), 50 absorbance (260 nm) units of tRNA plus 3 mM ATP.

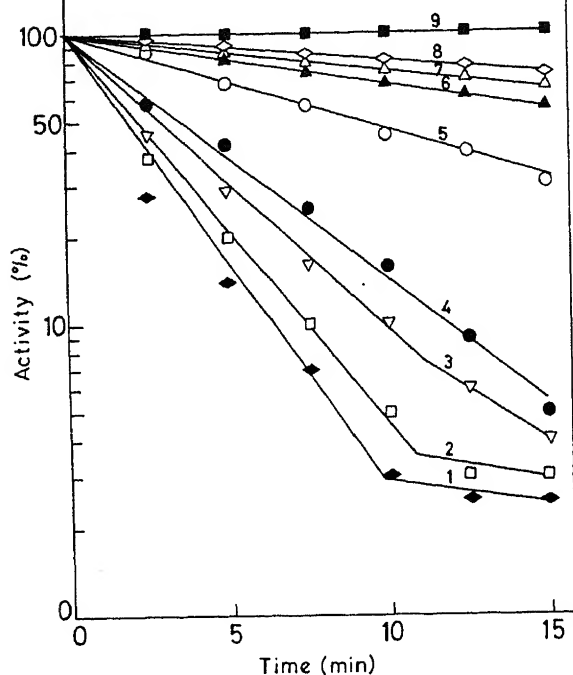
insight into the thermal characteristics of the enzyme. The enzyme was heated at 50°C, the optimum growth temperature of the organism, with the substrates and spermine individually and in combination for various time intervals. Aliquots were withdrawn and assayed at 40°C as mentioned earlier. The results of these experiments confirmed the earlier finding that substrates could protect the enzyme against heat denaturation to varying degrees. When present together tRNA, ATP and spermine could impart complete protection for the duration of the incubation (figure 4).

### Protein turnover

Rapid turnover of thermolabile macromolecules has been postulated as one of the mechanisms for growth at higher temperatures (Singleton and Amelunxen, 1973). In order to find out whether such a mechanism exists in *H. lanuginosa* the rate of protein turnover was studied by the double isotope method of Arias *et al.* (1969) as indicated under 'materials and methods'. The cells were grown at temperatures 40, 45, 50 and 55°C. The growth rate during the log phase at these temperatures was almost identical (data not presented). But the turnover rate was different at different temperatures. As the temperature of growth increased the protein turnover rate increased (table 1). This suggested that rapid turnover of proteins is also a mechanism operating in the fungus to overcome the ill effects of higher temperatures.

### Discussion

Results of the present studies show that the tRNA of this fungus is thermo-stable up to the optimum temperature of its growth in the presence of small



**Figure 4.** Rate of thermal denaturation of valine tRNA synthetase<sub>2</sub> at 50°C.

The enzyme was heated in the buffer at 50°C along with the effector and aliquots were withdrawn at definite time periods, chilled in ice and assayed at 40°C.

(1), No addition; (2), 180  $\mu$ M L-Valine; (3), 0.25 mM spermine; (4), 3 mM ATP; (5), 180  $\mu$ M L-Valine plus 3 mM ATP; (6), 50 absorbance (260 nm) units of tRNA; (7), tRNA plus 0.25 mM spermine; (8), tRNA plus 3 mM ATP; (9), tRNA plus 3 mM ATP plus 0.25 mM spermine.

**Table 1.** Protein turnover at different temperatures.

Growth temperature	$[^3\text{H}]/[^{14}\text{C}]$
40	1.34
45	1.34
50	2.1
55	4.7

*H. lanuginosa* cells were grown at a definite temperature and they were labelled with  $[^{14}\text{C}]$ -L-leucine for 30 min. After 2 h chase in the cold medium the cells were again labelled, this time with  $[^3\text{H}]$ -leucine, for 10 min as described under 'methods'. The labelled cells were extracted with buffer and the radioactivity in an aliquot of the protein fraction was determined.  $[^{14}\text{C}]$ - and  $[^3\text{H}]$ -counts are expressed as a

biological systems. It is not known whether the fungus contains spermine. The experiments, however, suggest that the intracellular environment can alter the stability of the tRNA. No appreciable melting occurs upto 55°C. However, *H. lanuginosa* tRNA apparently has no special thermal stability features different from that of the mesophilic yeast, *C. utilis*. The fact that the tRNA of *H. lanuginosa* is more resistant to the action of RNase A (figure 1B) shows that it is more compact.

The preliminary results obtained with the total aminoacyl tRNA synthetases (figure 2) when viewed in the light of the thermal characteristics of purified valine tRNA synthetase 2 (figures 3 and 4) suggest that other enzymes also of this fungus may not be very thermostable. They require stabilisation *in vivo* by various cellular factors. The overall picture that emerges from this study is that, unlike thermophilic bacteria, the protein synthesizing machinery of *H. lanuginosa* in general does not possess the intrinsic thermostability required for growth at higher temperatures. It is stabilised by interaction with magnesium ions, ATP, tRNA, polyamines and other cellular factors. The increase in the rate of protein turnover with growth temperature (table 1) suggests that optimum levels of some of the proteins are maintained by this mechanism also. Thus it appears that the presence of intrinsically thermostable proteins is not very crucial for thermophilic growth in fungi. Unlike bacteria these organisms do not grow at very high temperatures. During evolution the requirement for highly thermostable macromolecules may not have been stringent as in the case of bacteria. Stability acquired through alterations in the microenvironment of the cell and interaction with protective agents and also to some extent increased turnover appear to play more important roles as mechanisms to counteract the detrimental effects of higher temperature in *H. lanuginosa*.

## Acknowledgement

This project was supported in part by the University Grants Commission, New Delhi.

## References

- Amelunxen, R. E. and Murdock, A. L. (1978) *CRC Critical Rev.*, **6**, 343.
- Arias, I. M., Doyle, D. and Schimke, R. T. (1969) *J. Biol. Chem.*, **244**, 3303.
- Bellamy, A. R. and Ralph, R. K. (1968) *Methods Enzymol.*, **B12**, 156.
- Harris, C. L. (1978) *Nucleic Acids Res.*, **5**, 599.
- Ljungdahl, L. G. and Sherod, D. (1976) in *Extreme Environments, Mechanisms of Microbial Adaptation*. (ed. M. R. Heinrich) (New York: Academic Press) p. 147.
- Loginova, L. G. and Tashpulatov, Zh (1967) *Microbiology (Washington)*, **36**, 828.
- Muench, K. K. and Berg, P. (1966) *Procedures Nucleic Acid Res.* Vol. 1, (eds G. L. Cantoni and D. R. Davis) (Harper and Row) p. 375.
- Prasad, A. R. and Maheshwari, R. (1978a) *Biochim. Biophys. Acta.*, **525**, 162.
- Prasad, A. R. and Maheshwari, R. (1978b) *Proc. Indian Acad. Sci.*, **B87**, 231.
- Rao, B. V., Maheshwari, R., Sastri, N. V. S. and Subba Rao, P. V. (1979) *Curr. Sci.*, **48**, 113.
- Rao, B. V., Sastri, N. V. S. and Subba Rao, P. V. (1981) *Biochem. J.*, **193**, 389.
- Singleton, R. Jr. and Amelunxen, R. E. (1973) *Bacteriol. Rev.*, **37**, 320.
- Sumner, J. L. and Morgan, E. D. (1969) *J. Gen. Microbiol.*, **59**, 215.
- Sumner, J. L., Morgan, E. D. and Evans, H. C. (1969) *Can. J. Microbiol.*, **15**, 515.
- Tansey, M. R. and Brock, T. D. (1978) in *Microbial Life in Extreme Environments* (ed. D. J. Kushner)

- von der Haar (1976) *Biochem. Biophys. Res. Commun.*, **70**, 1009.
- von der Haar (1979) *Methods Enzymol.*, **59**, 257.
- von Ehrenstein, G. (1967) *Methods Enzymol.*, **A12**, 588.
- Wali, A. S., Mattoo, A. K. and Modi, V. V. (1979) *Int. J. Peptide Protein Res.*, **14**, 99.

## Site-directed mutagenesis to determine essential residues of ribulose-bisphosphate carboxylase of *Rhodospirillum rubrum*

SALIL K. NIYOGI, THOMAS S. SOPER, ROBERT S. FOOTE, FRANK W. LARIMER, RICHARD J. MURAL, SANKAR MITRA, EVA H. LEE, RICHARD MACHANOFF, and FRED C. HARTMAN

From the Protein Engineering and Molecular Mutagenesis Program of the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA

**Abstract.** Both Lys-166 and His-291 of ribulosebisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* have been implicated as the active-site residue that initiates catalysis. To decide between these two candidates, we resorted to site-directed mutagenesis to replace Lys-166 and His-291 with several amino acids. All 7 of the position-166 mutants tested are severely deficient in carboxylase activity, whereas the alanine and serine mutants at position 291 are ~40% and ~18% as active as the native carboxylase, essentially ruling out His-291 in the *Rhodospirillum rubrum* carboxylase (and by inference His-298 in the spinach enzyme) as a catalytically essential residue. The ability of some of the mutant proteins to undergo carbamate formation or to bind either ribulosebisphosphate or a transition-state analogue remains largely unimpaired. This implies that Lys-166 is not required for substrate binding; rather, the results corroborate the earlier postulate that Lys-166 functions as an acid-base group in catalysis or in stabilizing a transition state in the reaction pathway.

**Keywords.** Ribulose-P<sub>2</sub> carboxylase; site-directed mutagenesis; essentiality of Lys-166; non-essentiality of His-291.

### Introduction

In recent years, site-directed mutagenesis of proteins, or protein engineering, has developed into a powerful tool for studies of structure-function relationships of proteins. Among the various techniques of site-directed mutagenesis (Smith, 1985), the use of oligonucleotide-directed mutagenesis has provided a general method for producing desired mutations, such as base substitutions, deletions and insertions, at specific locations of the gene thereby leading to specific amino acid changes at desired sites of the protein under study (Smith and Gillam, 1981). Of the oligonucleotide-directed mutagenesis protocols, the most versatile is based on the use of a circular single-stranded (ss) DNA vector (usually M13 DNA) containing the target gene sequence and a synthetic DNA oligonucleotide containing the desired mutation to prime the synthesis *in vitro* of the complementary strand (Hutchison *et al.*, 1978; Razin *et al.*, 1978; Gillam and Smith, 1979a,b; Zoller and Smith, 1982, 1983).

Following transformation of competent cells with the resulting heteroduplex (usually enriched by band sedimentation in alkaline sucrose) containing the incorporated oligonucleotide, semi-conservative replication *in vivo* leads to the formation of homoduplexes derived from mutant and parental strands. Preliminary

screening for mutants is usually done by differential annealing with radioactively labelled mutagenic oligonucleotide, based on the substantial difference in the melting temperature of a perfectly matched duplex and that of a mismatched one (Wallace *et al.*, 1979). A convenient secondary screening often takes advantage of either the introduction of a new restriction endonuclease site or removal of an existing one. Final confirmation is obtained by direct sequence determination of the region encompassing the desired mutation.

D-ribulose 1,5-bisphosphate carboxylase/oxygenase (Ribulose-P<sub>2</sub> carboxylase) (EC, 4.1.1.39), which catalyzes the carboxylation of ribulose-P<sub>2</sub> to yield two molar equivalents of D-3-phosphoglycerate, is ubiquitous to photosynthetic organisms and provides the only significant route by which atmospheric CO<sub>2</sub> is converted to carbohydrate and therefore is absolutely essential to all higher forms of life. The enzyme also catalyzes an energy-wasteful, physiologically nonessential oxygenation reaction which competes with CO<sub>2</sub> utilization (see Lorimer, 1981; Miziorko and Lorimer, 1983, for reviews). Hence, an important long-term goal of our programme is to attempt alteration of the enzyme's substrate specificity to favor carboxylase activity, thereby increasing agricultural yields.

Active-site characterization is a key step in the elucidation of any enzyme mechanism. For ribulose-P<sub>2</sub> carboxylase, the complex yet well-established reaction pathway (figure 1) entails (a) enolization of ribulose-P<sub>2</sub> *via* abstraction of its C3 proton, (b) stereospecific carboxylation of the enediol intermediate, (c) hydration and carbon-carbon scission of the resultant 2-carboxy-3-keto intermediate, and (d) inversion and protonation of the aci-acid of 3-phosphoglycerate to complete product formation (Miziorko and Lorimer, 1983; Lorimer *et al.*, 1984; Jaworowski and Rose, 1985; Pierce *et al.*, 1986). The essential base initiating catalysis in step (a) has a pK<sub>a</sub> of 7.5 as established by the pH dependence of  $V_{\max}$  and the pH dependence of the deuterium isotope effect with [3-<sup>2</sup>H]ribulose-P<sub>2</sub> as substrate (Van Dyk and Schloss, 1986).

The use of affinity labels has proved invaluable in mapping and characterizing the active sites of ribulose-P<sub>2</sub> carboxylase (see Hartman *et al.*, 1984, for a review). The structural complexity of ribulose-P<sub>2</sub> carboxylase and lack of absolute specificity of the affinity labels used prompted the reliance on comparative amino acid sequence analysis to reveal whether residues implicated at the active-site by affinity labelling are indeed species invariant and thus likely to be essential to function (Hartman *et al.*, 1984; Nargang *et al.*, 1984). Because of their evolutionary diversity and structural dissimilarities, the carboxylases from spinach and *Rhodospirillum rubrum* (a purple non-sulphur photosynthetic bacterium) provide a stringent test of structural conservation. The quaternary structure of ribulose-P<sub>2</sub> carboxylase from spinach is typical among all higher plant and most bacterial carboxylases in being a hexadecamer with 8 large (53,000-Da) and 8 small (14,000-Da) subunits (Rutner, 1970; Martin, 1979; Zurawski *et al.*, 1981), whereas the functionally analogous enzyme from *R. rubrum* is a homodimer of 53,000-Da subunits (Tabita and McFadden, 1974; Schloss *et al.*, 1982; Hartman *et al.*, 1984). Moreover, compared to the > 80% sequence homology among most ribulose-P<sub>2</sub> carboxylases (Miziorko

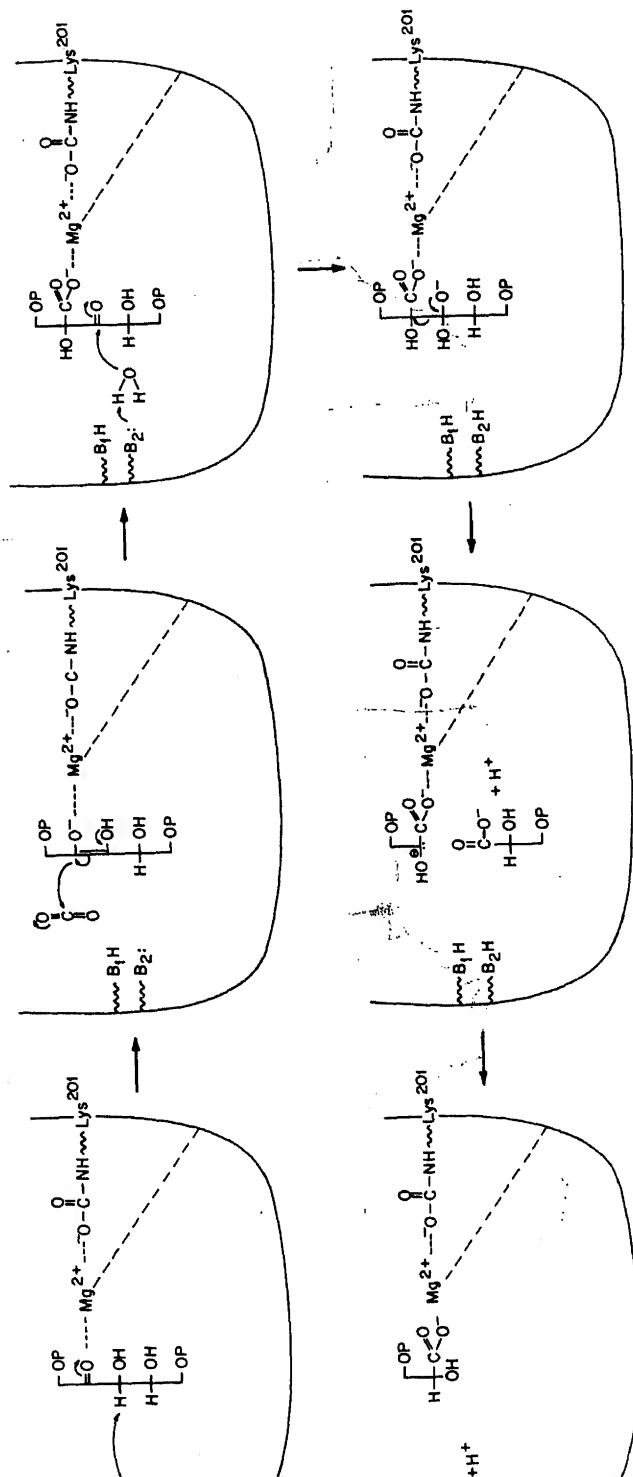
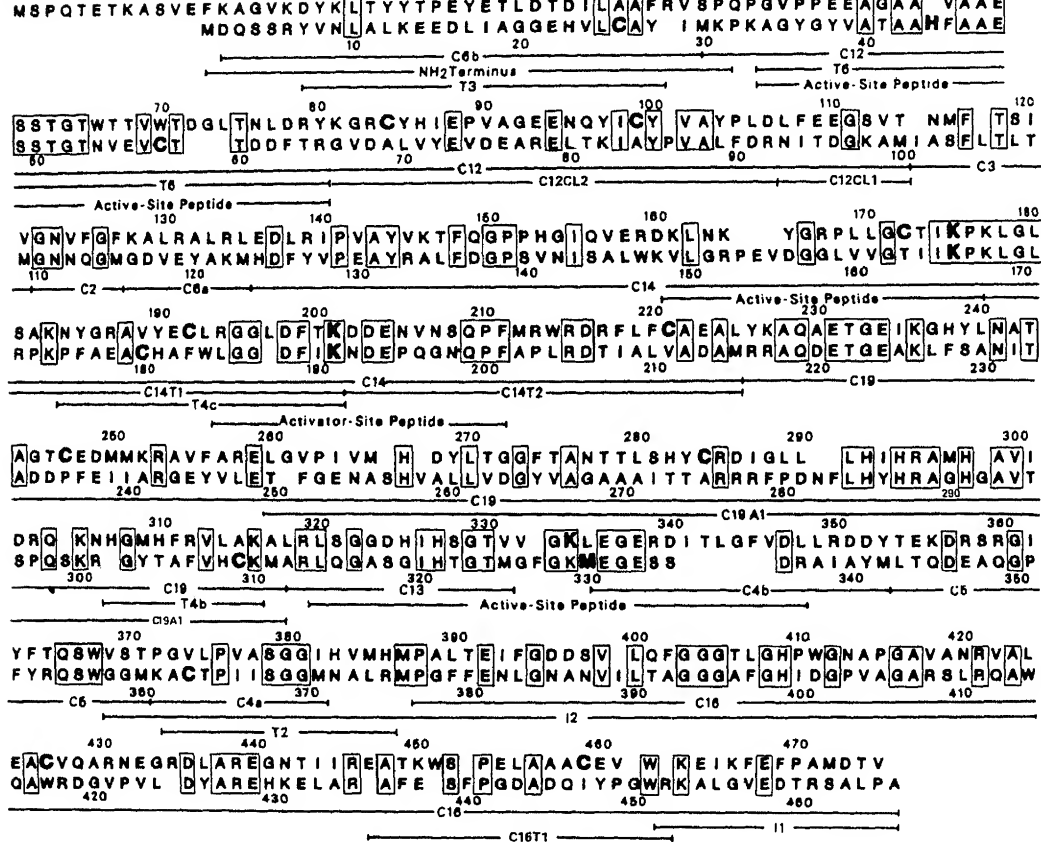


Figure 1. Reaction pathway for carboxylation of ribulose- $P_2$  as catalyzed by ribulose- $P_2$  carboxylase (Miziorko and Lorimer, 1983; Jaworowski and Rose, 1985). Reprinted from Hartman *et al.* (1986), with permission of the publisher.





**Figure 2.** Amino acid sequences of ribulose-P<sub>2</sub> carboxylase from spinach (upper) (Zurawski *et al.*, 1981) and *R. rubrum* (lower) (Hartman *et al.*, 1984; Nargang *et al.*, 1984). Alignments were made by visual inspection. Residues identical in both enzymes are enclosed in boxes. Gaps, attributed to deletions or insertions, appear as blank spaces. Peptide notations are explained in Hartman *et al.* (1984). Cysteine residues and residues identified at the active site or activator site by selective chemical labelling are illustrated with larger type. (Abbreviations: Asp, D; Asn, N; Glu, E; Gln, Q; Thr, T; Ser, S; Pro, P; Gly, G; Ala, A; Cys, C; Val, V; Met, M; Ile, I; Leu, L; Tyr, Y; Phe, F; His, H; Lys, K; Trp, W; Arg, R). Reprinted from Hartman *et al.* (1984), with permission of the publisher.

Based on studies mentioned above, both Lys-175 and Lys-334 in the spinach enzyme, which correspond to Lys-166 and Lys-329 in the *R. rubrum* enzyme, appear essential for enzyme activity. The extreme nucleophilicities and acidities of the  $\epsilon$ -amino group of Lys-166 ( $pK_a \sim 7.9$ ) of the *R. rubrum* enzyme and Lys-334 ( $pK_a$  of 9.0) of the spinach enzyme, as revealed by the pH dependencies of inactivations of the two enzymes by the lysine-selective reagent trinitrobenzene sulphonate, further support the postulate that both are important to catalysis rather than to substrate binding (Hartman *et al.*, 1985). Lys-166 may correspond to the essential base that enolizes ribulose-P<sub>2</sub> to initiate catalysis and that displays a  $pK_a$  of 7.5 in the pH

and McFadden, 1982) and the demonstration that the loss of activity correlated with modification of this residue (Igarashi *et al.*, 1985). A study of the pH dependence of enzyme inactivation by diethyl pyrocarbonate led to the suggestion that His-298 reflected an essential base ( $pK_a \sim 6.8$ ) observed in the pH profile for  $V_{max}$  (Paech, 1985).

It appeared unlikely that both a lysyl and an histidyl residue serve to abstract the C3 proton from ribulose-P<sub>2</sub>. Both, however, could be essential acid/base groups, since discrete steps in the overall reaction pathway (figure 1) entail proton transfers. In order to clarify the suggested roles of Lys-166 and His-291 in enzyme catalysis, we resorted to site-directed mutagenesis of the *R. rubrum* gene encoding ribulose-P<sub>2</sub> carboxylase at these two sites.

## Experimental Procedures

### Materials

**Chemicals and Enzymes:** These have been described earlier (Niyogi *et al.*, 1986).

**Strains, plasmids, and phage:** *Escherichia coli* JM107 [ $\Delta(lac-pro)$ , *end A1*, *gyrA96*, *thi-1*, *hsdR* 17, *supE44*, *relA1* (F' *traD36*, *proAB*<sup>+</sup>, *lacI $\phi$ Z*  $\Delta$ M15)] (Yanisch-Perron *et al.*, 1985) was used as the host for M13 vectors and for expression of the *rbc* gene. *E. coli* BNN45 (*metB*, *thi*<sup>-</sup>, *hsdR*, *lacY*, *supE44*, *supF*), derived from ED8654 (Murray *et al.*, 1977), was used as a cloning host.

The construction of plasmid pFL34 containing the *rbc* gene which expresses wild-type promoter has been described (Larimer *et al.*, 1986). The 1546-base pair *Bam*HI fragment containing the *rbc* gene was transferred into M13 mp19 vector (pFL19) in the orientation such that the ss phage DNA contains the strand complementary to the mRNA of the *rbc* gene. The phage DNA was purified from CsCl-banded phage as described earlier (Niyogi and Mitra, 1978).

### Methods

**Synthesis of Oligonucleotides:** Oligonucleotides (figure 3) used to produce mutations at the 166 and 291 sites in the *rbc* gene were synthesized by the phosphoramidite method (Sinha *et al.*, 1984) using a Systec model 1450 automated DNA synthesizer and were purified by electrophoresis on 20% polyacrylamide gels containing 8M urea (Atkinson and Smith, 1984).

**Oligonucleotide-directed mutagenesis of the *rbc* gene:** This was done as described before (Niyogi *et al.*, 1986) with some modifications. For annealing (in the single primer extension method—Smith, 1985; Nisbet and Beilharz, 1985; Niyogi *et al.*, 1986) the phosphorylated oligonucleotide (20 pmol) was heated with pFL19 DNA (1 pmol) for 10 min at a temperature 5–10°C higher than the empirically calculated temperature of dissociation (Td) (Meinkoth and Wahl, 1984), then at 10°C below the

A.

G T I I K P K L G L R  
 5'-GGACGATCATCAAGCCGAAGCTCGGCCTGCGT-3'  
 3'-CCGTGCTAGTAGTTCGGCTTCGAGCCGACGCA-5'

Mutant	Number	Oligonucleotide sequence	Strand	Monitored Restriction Site
Ala-166	1	5'-GATCATC <u>CGC</u> CGAAGCTTGGCCTGC-3' **	+	HindIII (+)
Arg-166	2	5'-TCATCAGGCGAAGCTTGGCCT-3' *	+	HindIII (+)
Cys-166	3	5'-CACGATCATCTGCGCGAAGCTCGGCCTGCGT-3' ***	+	HindIII (-)
Cys-166	4	3'-CATGGTAGTAGACGGGCTTCGA-5' ***	-	
Gln-166	5	3'-CATGCTAGTAGTTCGGCTTCGA-5' *	-	KpnI (-)
Gly-166	6	3'-CATGGTAGTAGCGGGCTTCGA-5' ***	-	
His-166	7	5'-GATCATCCACC CGAAGCTTGGCCT-3' **	+	HindIII (+)
Ser-166	8	5'-ATCATCAGCCCGAAGC-3' **	+	

B.

R A G H G A V  
 5'-CGGGCTGGCCAGCGCGCGTC-3'  
 3'-GCCCGACCGTCCGCGGCAG-5'

Mutant	Number	Oligonucleotide sequence	Strand	Monitored Restriction Site
Ala-291	I	5'-CGGGCTGGCGCGCGCGTC-3' **	+	BalI (-)
Ser-291	II	5'-CGGGCTGGCAAGCGCGCGTC-3' **	+	BalI (-)
Leu-291	III	5'-CGGGCTGGCCTCGCGCGTC-3' *	+	BalI (-)
Lys-291	IV	5'-CGGGCTGGCAAGCGCGCGTC-3' **	+	BalI (-)
Arg-291	V	5'-CGGGCTGGCCGCGCGCGTC-3' *	+	BalI (-)

Figure 3. Nucleotide sequences and encoded protein sequences of the *rbc* gene in the vicinity of the codon for (A) Lys-166 and (B) His-291, and relevant features of oligonucleotides used to introduce amino acid substitutions at these positions. The position-166 and position-291 codons or anticodons are underlined and the base substitutions which

10 for 15 min, followed by cooling at room temperature for 1 min. The yields of RF I DNA ranged between 5–50% of the input ssDNA template, depending on the choice of replication protocol and the (G + C) content of the oligonucleotide site (discussed later). Aliquots of RF I DNA were used to transfect *E. coli* JM107 according to the procedure of Hanahan (1985).

**Screening and characterization of mutants:** Plaque hybridization (Norrander *et al.*, 1983) with appropriate [<sup>32</sup>P]-labelled oligonucleotides as probes was performed for 1 h at a temperature 20°C below the empirically calculated T<sub>d</sub> (Meinkoth and Wahl, 1984), then washing at an appropriate temperature to discriminate between wild type and mutant phages, followed by autoradiography (Niyogi *et al.*, 1986). The candidate mutant phages from individual plaques were then grown, and RF DNA was isolated by the alkali lysis procedure (Maniatis *et al.*, 1982). Further screening was performed by appropriate restriction endonuclease treatments (see 'results' section). Mutations were finally confirmed by direct sequencing of ss phage DNA by the dideoxynucleotide chain termination protocol (Sanger *et al.*, 1977) using appropriate primers upstream of the target sequences.

**Expression and induction of mutant carboxylases:** Fragments harboring mutations generated by single primer extension were obtained by appropriate restriction endonuclease treatment of the corresponding RF I DNAs and subcloned into expression vectors. Mutants were also generated by 'bandaid mutagenesis'\* directly into the expression vectors. Such mutations were screened by restriction enzyme analysis and confirmed by sequencing of double-stranded plasmid DNA (Chen and Seeburg, 1985; Zagursky *et al.*, 1985). The induction by isopropylthiogalactoside and expression of normal or mutant carboxylase have been described (Niyogi *et al.*, 1986). Purification to near homogeneity, by immunoaffinity chromatography, of normal and mutant carboxylases from *E. coli* cell extracts has been described earlier (Niyogi *et al.*, 1986).

**Protein and enzyme assays:** Protein concentrations were determined according to Bradford (1976). Carboxylase and oxygenase activities were determined according to Lorimer *et al.*, (1977). Quantitation of carboxylase protein by dot immunobinding was performed as described earlier (Niyogi *et al.*, 1986).

## Results

### *Use of restriction enzyme digestion for additional screening*

As evident from figure 3, three of the mutants used in these studies, namely, Cys-166, Gln-166, and Gly-166, were introduced by bandaid mutagenesis. Silent mutations

---

\*Bandaid mutagenesis (Mural and Foote, 1986), independently reported as notch cloning (Childs *et al.*, 1985), uses a ss oligodeoxynucleotide, which codes for the desired mutation, to span a gap in the target gene which is created by digestion of the plasmid with two restriction endonucleases, one of which leaves a 3' protruding end while the other leaves a 5' protruding end. Since this technique involves a gap in the

the wild-type, in Ala-166, Arg-166, and His-166. For the His-291 site, a mutation leading to an amino acid substitution at that site also causes loss of the *Bal*I restriction site normally present in that region. These alterations provided convenient additional screening, besides differential hybridization, of potential mutants. Final confirmation of mutants was always obtained by direct sequence analysis of the mutant DNA.

As seen in figure 3, the Cys-166 mutation was generated by both the bandaid protocol and by the single primer method. No differences were found between these two independently derived mutant proteins.

### *Purity and quaternary structure of the mutant proteins*

The mutant proteins, isolated by immunoaffinity chromatography, were >90% pure. The almost identical electrophoretic mobilities of the native and mutant proteins on nondenaturing gels suggest that the mutants are also dimeric like the native enzyme (Niyogi *et al.*, 1986; Hartman *et al.*, 1987). Gel filtration on Sephadex G-150 further confirmed this conclusion (data not shown). Gel electrophoresis in the presence of sodium dodecyl sulphate showed that the mutant proteins are physically indistinguishable from wild-type enzyme (Niyogi *et al.*, 1986; Hartman *et al.*, 1987).

### *Enzymatic properties of mutant carboxylases*

Based on enzyme assays with purified mutant proteins, it is clear that the position-166 mutants either lack or display very low levels of carboxylase activity (table 1). None of the mutant proteins show detectable oxygenase activity (results not shown). It was possible to determine the  $K_m$  and  $k_{cat}$  of the serine mutant: these values are 35  $\mu$ M and 0.0072  $s^{-1}$  (Hartman *et al.*, 1987) compared to 6  $\mu$ M and 3.6  $s^{-1}$  for wild-type enzyme (Niyogi *et al.*, 1986). The catalytic efficiency ( $k_{cat}/K_m$ ) of the serine mutant is therefore only 0.03% of that of the wild-type enzyme.

Among the position-291 mutants, Ala-291 and Ser-291 display considerable levels of carboxylase activity (table 1). The oxygenase activities of these mutants were correspondingly reduced in comparison to the native enzyme (results not shown). The lowered specific activity of the alanine mutant reflected both a decreased  $k_{cat}$  and an increased  $K_m$ , with ribulose- $P_2$  as the substrate: these values are 1.5  $s^{-1}$  and 90  $\mu$ M compared to 3.6  $s^{-1}$  and 6  $\mu$ M for the native enzyme (table 1; Niyogi *et al.*, 1986). Hence, the catalytic efficiency of the alanine mutant is about 27% of that of the native enzyme.

### *Carbamylation and complexation of mutant carboxylases with carboxyarabinitol- $P_2$*

The transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate (carboxyarabinitol- $P_2$ ) is a tight-binding inhibitor of  $CO_2/Mg^{2+}$ -activated (*i.e.*, carbamylated), native carboxylase and forms a stable quaternary complex that is isolable by gel

Table 1. Enzymatic properties of mutant proteins.

Amino acid substitution	Specific activity (% wild-type)	$K_m$ (ribulose-P <sub>2</sub> )	Carbamate formation (mol/mol subunit)	Carboxy-arabinitol-P <sub>2</sub> binding (mol/mol subunit)
A. Mutations at Lys-166 Site				
Lys (wild-type)	100	6 $\mu$ M	1.0	1.0
Ser	0.2	35 $\mu$ M	0.6	0.8
Ala	0.1		0.4	0.6
Arg	0.02		ND	ND
Gln	$\leq 0.01$		ND	ND
His	$\leq 0.01$		ND	ND
Cys	$\leq 0.01$		ND	ND
Gly	$\leq 0.001$		0.5	0.5
B. Mutations at His-291 Site				
His (wild-type)	100	6 $\mu$ M	1.0	1.0
Ala-291	40	90 $\mu$ M	0.9	0.9
Ser-291	18			0.8
Leu-291	0.9		0.8	0.6
Lys-291	0.05		0.9	0.8
Arg-291	$\leq 0.01$			

ND indicates 'not detectable' under the assay conditions. A blank space indicates that no determination was made.

filtration (Miziorko and Sealy, 1980). Separate samples of each mutant protein were incubated in the presence of  $Mg^{2+}$  with either  $H^{14}CO_3^-$  and unlabelled carboxy-arabinitol-P<sub>2</sub> or unlabelled  $HCO_3^-$  and [ $^{14}C$ ]-carboxyarabinitol-P<sub>2</sub>; these samples were then individually subjected to gel filtration.

With the Arg, Gln, His, and Cys mutants at position-166, binding of neither  $^{14}CO_2$  nor [ $^{14}C$ ]-carboxyarabinitol-P<sub>2</sub> is detected. However, with the Ser, Ala and Arg mutants at position-166 and the Ala, Ser, Leu and Lys mutants at position-291, quaternary complexes are formed, as determined with either  $^{14}CO_2$  or [ $^{14}C$ ]-carboxyarabinitol-P<sub>2</sub> (Hartman *et al.*, 1987; Niyogi *et al.*, 1986). The observed stoichiometries of binding  $CO_2$  or carboxyarabinitol-P<sub>2</sub> are listed in table 1. When the complexes of the position-166 mutant proteins with [ $^{14}C$ ]-carboxyarabinitol-P<sub>2</sub> are challenged with a 10-fold molar excess of unlabelled carboxyarabinitol-P<sub>2</sub> prior to gel filtration, the levels of protein-bound radioactivity were reduced 10-fold. Similar results were obtained with the Ala-291 mutant. These results suggest complete ligand exchange. Under identical conditions, no exchange of ligands takes place with the quaternary complex prepared from wild-type enzyme (Hartman *et al.*, 1985).

## Discussion

Because of our long-term goal of applying site-directed mutagenesis to different

single-primer extension in the M13 system (Zoller and Smith, 1983). However, satisfactory yields of replication form (RF) I DNA, a crucial requirement for the subsequent transfection step, were obtained only upon modification of published reaction protocols (Nisbet and Beilharz, 1985; Zoller and Smith, 1983). For example, the pre-melting step (an addition to the usual protocol) and the annealing reaction were performed in the absence of  $MgCl_2$  and dithiothreitol—usual reaction constituents—and at temperatures that reflected the Td of the particular oligonucleotide-M13 ssDNA complex. These and other modifications and the choice of the temperature and enzyme concentrations during the replication/ligation reaction (Niyogi, S. K. and Yette, M. L., unpublished results) were designed to overcome the formation of hairpin structures in ssDNA due to self-pairing (Niyogi and Mitra, 1978). The high (G + C) contents, 65% for the entire *rbc* gene, 55–65% for the 16–31-nucleotide region surrounding the Lys-166 codon, and 90% for the 21-nucleotide region surrounding the His-291 region, are particularly conducive to the formation of hairpin structures. These could presumably interfere with the annealing of the mutagenic oligonucleotide and also inhibit the elongation process during the replication reaction. The potential advantages of using bandaid mutagenesis in circumventing problems encountered during *in vitro* replication and in the facile introduction of multiple mutations at a given site will be detailed in a future publication.

Site-directed mutagenesis was employed to determine which of the two postulated amino acid residues, Lys-166 or His-291, may serve to abstract the C3 proton from ribulose- $P_2$ . The stringency of the requirement was evaluated by substitution with a number of different amino acids at each position. The rationale for choosing a particular amino acid is as follows: For Lys-166, (a) glycine, total removal of the lysyl side-chain; (b) alanine, substantial removal of the lysyl side-chain but less likely to introduce conformational changes in the polypeptide backbone; (c) serine and glutamine, to more closely approximate (in contrast to glycine and alanine) the hydrophilicity of lysine without increasing its steric bulk; (d) arginine, retention of a cationic side-chain; (e) cysteine and histidine, retention of acid/base groups for partial fulfillment of the function of the lysyl side-chain, in spite of possible perturbation caused by the heterocyclic ring of histidine. Similarly, a list of rationales can be generated for the substitutions for His-291.

The substantial activities of the Ala-291 and Ser-291 mutants (table 1) definitely exclude His-291 in the *R. rubrum* carboxylase (and by inference His-298 in the spinach carboxylase) as a catalytically essential residue. However, the decreased  $k_{cat}$  and increased  $K_m$  (for ribulose- $P_2$ ) of the Ala-291 mutant, in comparison to the corresponding parameters of the native enzyme, suggest that His-291 could be a residue in the active site region. Alternatively, the changes in the kinetic parameters could signify conformational changes brought about by substitution for a residue somewhat remote from the active site. Because of the extensive sequence homology of the regions encompassing His-291 in the *R. rubrum* enzyme and His-298 in the spinach enzyme (figure 2), the acknowledged universality of the carboxylase mechanism and the species invariance of residues assigned to the catalytic and activator sites by chemical modification data (Hartman *et al.*, 1984), one can also reasonably exclude His-298 in the spinach enzyme as a catalytically essential residue.

What about Lys-166 in the *R. rubrum* carboxylase (and by inference Lys-175 in the

pinach enzyme)? Since all 7 of the mutant proteins display very low levels or are devoid of carboxylase activity, Lys-166 appears to be essential for catalytic activity. However, the role in catalytic activity can hardly be due to substrate binding, since the  $K_m$  of the serine mutant (the most active among the mutants) for ribulose-P<sub>2</sub> is only 6 times greater than that observed with authentic *R. rubrum* carboxylase. The very low activity of the arginine mutant (a rather conservative replacement for lysine) also argues against a direct role of Lys-166 in substrate binding.

The ability of the Ala-166, Ser-166 and Gly-166 mutants to undergo carbamylation and subsequently bind the substrate analogue carboxyarabinitol-P<sub>2</sub> (table 1) clearly argues for the case that Lys-166 is not required for the activation process or for substrate binding. This argument becomes particularly convincing with values obtained with the glycine mutant (table 1) which is devoid of enzyme activity. Because of the proximity of the carbamate to the active site (Pierce and Reddy, 1986), one would have expected major perturbations of carbamate formation if Lys-166 was indeed intimately involved in the activation process.

Since Lys-166 is not essential for carbamate formation or substrate binding and given the observations that the position-166 mutants are similar to the native enzyme in terms of their dimeric structure and gross conformation, we are left with the conclusion that the  $\epsilon$ -amino group of Lys-166 serves as a general base (perhaps the one that enolizes ribulose-P<sub>2</sub>) or somehow stabilizes a transition state in the reaction pathway.

## Acknowledgements

We thank Alice A. Hardigree, Bruce E. Suttle and Margaret L. Yette for their expert technical assistance. Research sponsored jointly by the Office of Health and Environmental Research, US Department of Energy under contract DE-AC05-40R21400 with the Martin Marietta Energy Systems, Inc. and by the Oak Ridge National Laboratory Director's Research and Development Fund.

## References

- Atkinson, T. and Smith, M. (1984) in *Oligonucleotide Synthesis* (ed. M. J. Gait) (Oxford: IRL Press) p. 35.
- Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248.
- Chen, E. Y. and Seeburg, P. H. (1985) *DNA*, **4**, 165.
- Childs, J., Villanueva, K., Barrick, D., Schneider, T. D., Stormo, G. D., Gold, L., Leitner, M. and Caruthers, M. (1985) in *Sequence Specificity in Transcription and Translation* (eds R. Calendar and L. Gold) (New York: Alan R. Liss, Inc.) p. 341.
- Gillam, S. and Smith, M. (1979a) *Gene*, **8**, 81.
- Gillam, S. and Smith, M. (1979b) *Gene*, **8**, 99.
- Hanahan, D. (1985) *DNA Cloning* **1**, 109.
- Hartman, F. C., Milanez, S. and Lee, E. H. (1985) *J. Biol. Chem.*, **260**, 13968.
- Hartman, F. C., Soper, T. S., Niyogi, S. K., Mural, R. J., Foote, R. S., Mitra, S., Lee, E. H., Machanoff, R. and Larimer, F. W. (1987) *J. Biol. Chem.*, (in press).
- Hartman, F. C., Stringer, C. D. and Lee, E. H. (1984) *Arch. Biochem. Biophys.*, **232**, 280.
- Hartman, F. C., Stringer, C. D., Milanez, S. and Lee, E. H. (1987) *Philos. Trans. R. Soc. London B. Biol. Sci.* **B313**, 379.



- Jaworowski, A. and Rose, I. A. (1985) *J. Biol. Chem.*, **260**, 944.
- Larimer, F. W., Machanoff, R. and Hartman, F. C. (1986) *Gene*, **41**, 112.
- Lee, E. H., Stringer, C. D. and Hartman, F. C. (1987) *Proc. Natl. Acad. Sci. USA*, (in press).
- Lorimer, G. H. (1981) *Annu. Rev. Plant Physiol.*, **32**, 349.
- Lorimer, G. H., Badger, M. R. and Andrews, T. J. (1977) *Anal. Biochem.*, **78**, 66.
- Lorimer, G. H., Pierce, J., Gutteridge, S. and Schloss, J. V. (1984) in *Adv. in Photosynth. Res.*, **3**, 725.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, New York: Cold Spring Harbor Laboratory.
- Martin, P. G. (1979) *Aust. J. Plant Physiol.*, **6**, 401.
- Meinkoth, J. and Wahl, W. (1984) *Anal. Biochem.*, **138**, 267.
- Miziorko, H. M. and Lorimer, G. H. (1983) *Annu. Rev. Biochem.*, **52**, 507.
- Miziorko, H. M. and Sealy, R. C. (1980) *Biochemistry*, **19**, 1167.
- Mural, R. J. and Foote, R. S. (1986) *DNA*, **5**, 84.
- Murray, N. E., Brammer, W. J. and Murray, K. (1977) *Mol. Gen. Genet.*, **150**, 53.
- Nargang, F., McIntosh, L. and Somerville, C. (1984) *Mol. Gen. Genet.*, **193**, 220.
- Nisbet, J. T. and Beilharz, M. W. (1985) *Gene Anal. Tech.*, **2**, 23.
- Niyogi, S. K., Foote, R. S., Mural, R. J., Larimer, F. W., Mitra, S., Soper, T. S., Machanoff, R. and Hartman, F. C. (1986) *J. Biol. Chem.*, **261**, 10087.
- Niyogi, S. K. and Mitra, S. (1978) *J. Biol. Chem.*, **253**, 5562.
- Norrander, J., Kempe, T. and Messing, J. (1983) *Gene*, **26**, 101.
- Paech, C. (1985) *Biochemistry*, **24**, 3194.
- Pierce, J., Andrews, J. and Lorimer, G. H. (1986) *J. Biol. Chem.*, **261**, 10248.
- Pierce, J. and Reddy, G. S. (1986) *Arch. Biochem. Biophys.*, **245**, 483.
- Razin, A., Hiroshi, T., Itakura, K. and Riggs, A. D. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4268.
- Rutner, A. C. (1970) *Biochem. Biophys. Res. Commun.*, **39**, 923.
- Saluja, A. K. and McFadden, B. A. (1982) *Biochemistry*, **21**, 89.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463.
- Schloss, J. V., Phares, E. F., Long, M. V., Norton, I. L., Stringer, C. D. and Hartman, F. C. (1982) *Methods Enzymol.*, **90**, 522.
- Sinha, N. D., Biernat, J., McManus, J. and Koster, H. (1984) *Nucleic Acids Res.*, **12**, 4539.
- Smith, M. (1985) *Ann. Rev. Genet.*, **19**, 423.
- Smith, M. and Gillam, S. (1981) in *Genet. Eng. Principles Methods*, **3**, 1.
- Tabita, F. R. and McFadden, B. A. (1974) *J. Biol. Chem.*, **249**, 3459.
- Van Dyk, D. E. and Schloss, J. V. (1986) *Biochemistry*, **25**, 5145.
- Wallace, K. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T. and Itakura, K. (1979) *Nucleic Acids Res.*, **6**, 3543.
- Wells, J. A., Vasser, M. and Powers, D. B. (1985) *Gene*, **34**, 315.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, **33**, 103.
- Zagursky, R., Baumeister, N., Lomox, N. and Berman, M. (1985) *Gene Anal. Tech.*, **2**, 89.
- Zoller, M. J. and Smith, M. (1982) *Nucleic Acids Res.*, **10**, 6487.
- Zoller, M. J. and Smith, M. (1983) *Methods Enzymol.*, **100**, 468.
- Zurawski, G., Perrot, B., Bottomley, W. and Whitfield, P. R. (1981) *Nucleic Acids Res.*, **9**, 3251.

## Purification and some properties of human DNA-*O*<sup>6</sup>-methylguanine methyltransferase

AMY M. BOULDEN\*, ROBERT S. FOOTE, G. SCOTT FLEMING and SANKAR MITRA†

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, and

†Biology Division, Oak Ridge National Laboratory, P.O. Box Y, Oak Ridge, Tennessee 37831, USA

\*Present address: Centre College, Box 70, Danville, Kentucky 40422, USA

**Abstract.** DNA-*O*<sup>6</sup>-methylguanine methyltransferase was purified from the nuclear fraction of fresh human placenta using ammonium sulphate precipitation, gel filtration, affinity chromatography on DNA-cellulose and hydroxyapatite. The methyltransferase preparation was approximately 1-2% pure based on specific activity, and was free of nucleic acids. The protein reacts stoichiometrically with *O*<sup>6</sup>-methylguanine in DNA with apparent second-order kinetics. The human methyltransferase has a pH optimum of about 8.5, similar to that of the corresponding rat and mouse proteins. NaCl inhibits the reaction in a concentration-dependent fashion. The human protein, like the rodent and *E. coli* methyltransferases, needs no cofactor. While 1 mM MnCl<sub>2</sub>, 1 mM spermidine, 5 mM MgCl<sub>2</sub> and 10 mM EDTA individually do not significantly inhibit the initial rate of reaction, the protein is nearly completely inactive in 5 mM AlCl<sub>3</sub> or FeCl<sub>2</sub> or 10 mM spermidine. The initial rate of reaction increases as a function of temperature at least up to 42°. The reaction is inhibited by DNA in a concentration-dependent manner, with single-stranded DNA being more inhibitory than duplex DNA.

**Keywords.** DNA repair; DNA alkylation; *O*<sup>6</sup>-methylguanine; human methyltransferase.

## Introduction

A critical factor in cellular responses to chemical mutagens and carcinogens is the ability of the cells to repair damages in DNA resulting from its reaction with the xenobiotic agents or their metabolites. Among the DNA lesions produced by simple methylating agents, such as *N*-methyl nitrosamines, *O*<sup>6</sup>-methylguanine is of particular interest because of its inability to form normal base-pairs with cytosine (Loveless, 1969) and its preferential base-pairing with thymine during DNA replication (Snow *et al.*, 1984). Lack of *O*<sup>6</sup>-alkylguanine repair correlates with increased mutagenicity in *Escherichia coli* (Samson and Cairns, 1977; Jeggo *et al.*, 1977; Jeggo, 1979), and is a possible factor in the organ specificity of tumors induced by alkylating agents (Goth and Rajewsky, 1974; Nicoll *et al.*, 1975).

In both bacterial and mammalian cells, *O*<sup>6</sup>-methylguanine in DNA is repaired by similar methyltransferases; these proteins transfer the added methyl group to a cysteine residue on their own amino acid chains (Olsson and Lindahl, 1980; Mehta *et al.*, 1981; Bogden *et al.*, 1981; Pegg *et al.*, 1982) thereby restoring the original guanine base in DNA (Foote *et al.*, 1980). DNA-*O*<sup>6</sup>-methylguanine methyltransferase

same in *E. coli* and mammalian cells, the methyltransferases from these sources differ structurally and functionally. The protein is induced by 10- to 100-fold following exposure of *E. coli* to alkylating agents (Schendel and Robins, 1978; Karran, *et al.*, 1979; Robins and Cairns, 1979; Foote *et al.*, 1980; Mitra *et al.*, 1982). In contrast, the mammalian methyltransferases are only slightly inducible, if at all, by alkylation treatment (Montesano *et al.*, 1979; Sklar *et al.*, 1981; Myrnes *et al.*, 1982; Karran *et al.*, 1982; Foote and Mitra, 1984), and in the liver appear to respond to damage and regeneration in general, rather than specifically to alkyl-DNA lesions (Cleaver and Kaufmann, 1980). In *E. coli*, the methyltransferase activity for both *O*<sup>6</sup>-alkylguanine and *O*<sup>4</sup>-alkylthymine in DNA is present in the 39 kDa Ada protein (McCarthy *et al.*, 1984). An 18 kDa cleavage product retains the methyltransferase activity for *O*<sup>6</sup>-methylguanine (Teo *et al.*, 1984); the Ada protein also contains a second cysteine residue which accepts alkyl groups from methyl phosphotriesters in DNA (McCarthy and Lindahl, 1985; Margison *et al.*, 1985). Methylation of this second cysteine acceptor is responsible for the induction of methyltransferase in *E. coli* (Teo *et al.*, 1986). However, unlike the *E. coli* protein, the size of mammalian methyltransferases is approximately 24 kDa, and the mammalian proteins display no repair activity for phosphotriesters and *O*<sup>4</sup>-alkylthymine (Yarosh *et al.*, 1985; Pegg *et al.*, 1983; Dolan *et al.*, 1984).

Although the bacterial methyltransferase has been extensively studied, the mammalian methyltransferases in general, and the human enzyme in particular, have not been well-characterized. Human studies are, moreover, hampered by the relative difficulty of tissue acquisition. The enzyme has been purified from regenerating rat liver approximately 1300-fold, resulting in a 2% pure protein (Pegg *et al.*, 1983), and from normal rat liver with 3800-fold purification and at least 0.15% purity (Hora *et al.*, 1983). A 3-step purification of mouse liver yielding 86-fold purification has also been reported, with no estimate of purity level given (Bogden *et al.*, 1981). Yarosh *et al.* (1984) describe a partial purification of DNA-*O*<sup>6</sup>-methylguanine methyltransferase from human placenta, but the resulting fraction was less than 0.002% pure.

In this paper we describe an approximate 66,000-fold purification of DNA-*O*<sup>6</sup>-methylguanine methyltransferase from human placenta. An initial characterization of the partially purified protein is also reported.

## Materials and methods

### Materials

[CH<sub>3</sub>-<sup>3</sup>H]-*N*-Methyl-*N*-nitrosourea (4 Ci/mmol) was purchased from Moravsek Biochemicals Brea, California, USA. Calf thymus DNA was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. All other biochemical reagents were of reagent grade. Single-stranded DNA bound to CM-cellulose was prepared according to Potuzak and Wintersberger (1976).

### *Preparation of O<sup>6</sup>-methylguanine-DNA substrate*

DNA containing *O*<sup>6</sup>-[<sup>3</sup>H]-methylguanine was prepared by a modification of the procedure reported by Karran *et al.* (1979). Calf thymus DNA was treated with N-[<sup>3</sup>H]-methyl-*N*-nitrosourea for 18 h at 37°C, precipitated with 2 volumes ethanol, and incubated with 200 mM Na-cacodylate buffer, pH 7.2, at 80°C for 16 h to release 7- and 3-methylpurines. The preparation was first dialyzed at 4° for 48 h against 50 mM Tris-Cl, pH 7.4, and 1 mM EDTA with 1 M NaCl and then for 48 h against the same buffer without added NaCl. The final substrate had a specific radioactivity of about 100,000 cpm per mg DNA, of which 55% was present in *O*<sup>6</sup>-methylguanine as determined by acid hydrolysis and chromatography of an aliquot (Foote *et al.*, 1983). The remaining radioactivity was present primarily in phosphotriesters with a small amount in methylated pyrimidines. Only *O*<sup>6</sup>-methylguanine acts as a substrate for the methyltransferase.

### *Assay of DNA-O<sup>6</sup>-methylguanine methyltransferase*

Standard assay buffer, unless stated otherwise, contained 20 mM K-PO<sub>4</sub> (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 mM dithiothreitol (DTT), and 0.2 mM  $\alpha$ -toluenesulphonyl fluoride ( $\alpha$ ToSF). Protein samples were mixed with a solution of assay buffer containing 160 fmol *O*<sup>6</sup>-methylguanine in DNA and other components as indicated, in a total reaction volume of 200  $\mu$ l. The mixture was incubated at 37°C and the reaction was terminated at 1 h (unless otherwise noted) by the addition of sodium dodecylsulphate to a final concentration of 1%. Protein was degraded by the addition of 100  $\mu$ g Proteinase K (EC 3.4.21.14) (EM Biochemicals) and subsequent incubation at 37°C for 2 h. DNA was then precipitated by the addition of 0.3 M sodium acetate, 50  $\mu$ g calf thymus DNA as carrier, and 3 volumes of ethanol, followed by freezing in liquid nitrogen for 5 min. The precipitate was removed by centrifugation at 10,000 *g* for 5 min and the supernatant was assayed for radioactivity in the presence of 9 ml ACS scintillation fluid (Amersham). Incubations containing bovine serum albumin (1.2  $\mu$ g) in place of methyltransferase were used to determine background radioactivity (Waldstein *et al.*, 1982).

## **Results and discussion**

### *Purification of DNA-O<sup>6</sup>-methylguanine methyltransferase from human placenta*

In preliminary experiments, we found that whole extracts of human placenta contain activity equivalent to approximately 10  $\mu$ g of the protein (based on molecular weight of 24,000) per 100 g of tissue. Up to 90% of the activity could be recovered in a crude nuclear fraction with an approximate 10-fold purification. In order to accumulate sufficient material for extensive purification of the methyltransferase, we routinely processed fresh placentas (300–400 g) and stored the pelleted nuclear fraction at

**Table 1.** Summary of purification of DNA-*O*<sup>6</sup>-methylguanine methyltransferase from human placenta.

Step (Fraction No.)	Total A280 units	Total Activity (10 <sup>6</sup> cpm)	Fold purification	Yield (%)
Nuclear extract (I)	53,460	15	*10	[90*]
Polyethyleneimine precipitation (II)	3,380	9	94	54
DEAE cellulose with DNA cellulose (III)	111	1.5	470	8.7
0-70% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (IV)	103	1.8	610	11
Sephadex G-75 (V)	10.7	0.56	1,830	3.2
Hydroxyapatite (VI)	0.19	0.37	65,930	2.2

\*Estimate of purification from the crude extract based on previous studies.

from local hospitals as soon after delivery as possible and were transported to the laboratory on ice. All procedures were carried out at 0–4°C. After removal of the membrane, the tissue was cut into several small pieces and washed with 20 mM Tris-Cl (pH 7.4), 10% glycerol, 1 mM EDTA, 1 mM  $\beta$ -mercaptoethanol and 0.2 mM  $\alpha$ ToSF (buffer A) to remove excess blood. The tissue was then homogenized in two volumes of buffer A using a Polytron homogenizer (Brinkman Instruments) at low speed. The homogenate was strained through cheesecloth and the retained material was rehomogenized in one volume of buffer A and filtered again. The process was repeated once more and the combined filtrate was centrifuged at 6000 *g* for 30 min to pellet the intact and fragmented nuclei. The pellet was resuspended in one volume of buffer A and recentrifuged. The crude nuclear pellet could be stored at –80°C for up to several weeks without a significant loss of methyltransferase activity.

Several pellet fractions (equivalent to 2–3 placentas) were combined after suspension in an equal volume of buffer A (based on the original weight of tissue) and sonicated with 3 min bursts until no intact nuclei were detectable under the microscope. The extract was then centrifuged at 10,000 *g* for 30 min and the supernatant (Fraction I) was made up to 0.02% polyethyleneimine (BDH Chemicals). After stirring for 30 min, the precipitated nucleic acid was removed by centrifugation at 10,000 *g* for 30 min. The supernatant (Fraction II) was loaded at 2 ml/min on a DEAE-cellulose (DE52; 4.2 cm  $\times$  10 cm) column connected in tandem with a ssDNA-cellulose column (6.5 cm  $\times$  7.5 cm) pre-equilibrated with buffer A. After loading of the sample, the columns were washed with 2 litres of 50 mM NaCl in buffer A at 2 ml/min before the methyltransferase was eluted with 0.5 M NaCl in buffer A. Fractions of 7 ml were collected and assayed, and those containing methyltransferase activity were pooled (Fraction III). The protein was then precipitated by 70% saturation with ammonium sulphate and collected by centrifugation (10,000 *g*, 30 min). The pelleted protein was dissolved in buffer A to a final volume of up to 40 ml (Fraction IV) and applied to a Sephadex G-75 column (2.7 cm  $\times$  85 cm). The column was eluted with 0.2 M NaCl in buffer A (0.3 ml/min). Fractions of 3 ml were collected and those containing methyltransferase activity (eluting at 42–68% of the column volume) were pooled. The enzyme was then concentrated by ultrafiltration in an Amicon stirred cell using a YM 10 filter (Fraction V).

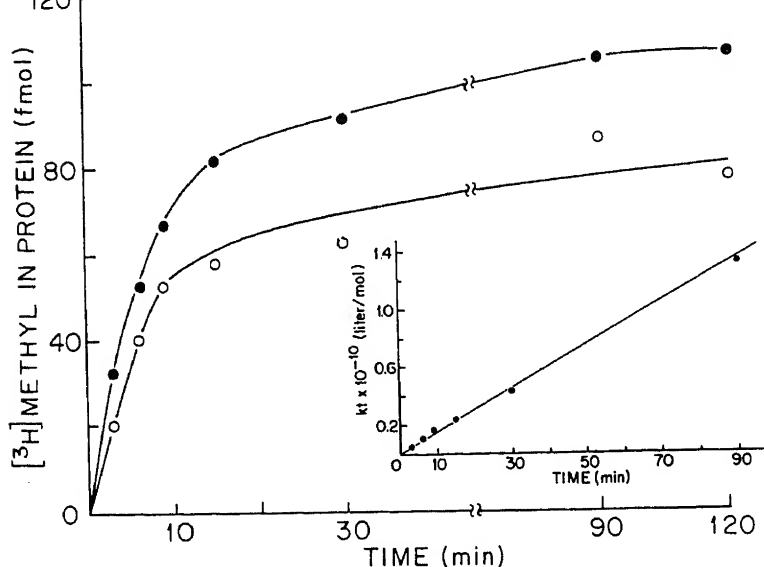
Protein concentration was estimated by absorbance values at 280  $\mu\text{m}$ . Based on the ratio of absorbance at 260 and 280  $\mu\text{m}$ , the enzyme preparation was completely free of nucleic acids after gel filtration. The hydroxyapatite fraction has an absorbance too low to be measured accurately but a rough calculation of the mass of methyltransferase, based on its activity and molecular weight of 24,000, and the concentration of protein, indicates that the final hydroxyapatite fraction was about 1–2% pure. Attempts to purify the protein further *via* additional methods including high performance liquid chromatography caused a drastic loss in its recovery. In any case, we believe that our methyltransferase is more pure than any other mammalian preparation reported in the literature so far.

### *Kinetic behavior of the methyltransferase*

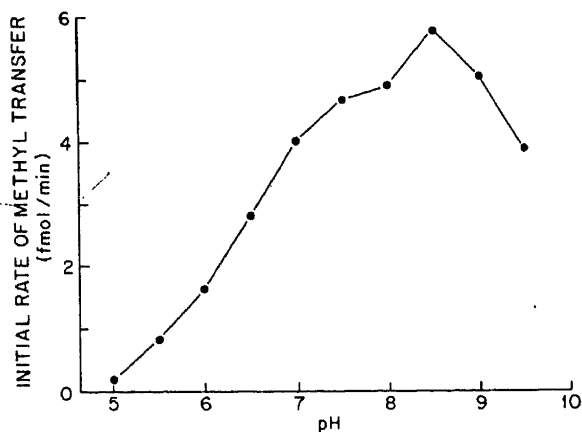
Crude extracts and partially purified DNA- $O^6$ -methylguanine methyltransferase from mammalian cells and tissues demethylate  $O^6$ -methylguanine in an apparently stoichiometric rather than catalytic fashion, as was shown with the *E. coli* enzyme (Foote *et al.*, 1980; Bogden *et al.*, 1981; Yarosh *et al.*, 1984; Hora *et al.*, 1983; Pegg *et al.*, 1983). However, the definitive evidence for a direct methyl transfer from  $O^6$ -methylguanine in DNA to the methyltransferase itself on an equivalent basis, as shown with the *E. coli* protein (Lindahl *et al.*, 1982), could not be provided without a homogeneous enzyme preparation. Nonetheless, we determined the rate of demethylation reaction with our purified enzyme (figure 1). The reaction follows apparent second-order kinetics (figure 1, inset) with a calculated rate constant of approximately  $1.5 \times 10^8 \text{ litre mol}^{-1} \text{ min}^{-1}$  (at 37°). Figure 1 also confirms the stoichiometric nature of reaction in that the extent of reaction at the plateau level corresponds to the amount of protein added.

### *pH-Dependence of the reaction rate of the methyltransferase*

As is evident from figure 1, the rate of methyl transfer declines after the first 10 min of incubation. The reason for this may be that, in addition to the depletion of the alkylated base in DNA and the methyltransferase, all  $O^6$ -methylguanines in DNA are not equally susceptible to the methyltransferase, possibly because of the effect of neighboring base sequences (Topal *et al.*, 1986). In any case, we routinely used only the initial rate for further kinetic studies by determining the extent of methylation after 3 and 6 min of incubation. The pH-dependence of the initial rate of reaction (figure 2) shows an optimum pH of about 8.5 for the human methyltransferase. This is comparable to the values obtained with other mammalian methyltransferases (Hora *et al.*, 1983). The *E. coli* methyltransferase also reacts optimally in slightly alkaline pH. While the pK of the sulphhydryl group of the acceptor cysteine residue on the protein is not known, these results are consistent with the idea that Cys-S<sup>-</sup> form is the reactive species and that the activity curve reflects the pH-dependent ionization of Cys-SH which has a pK of 8–8.9 in the free form (Sober, 1968).

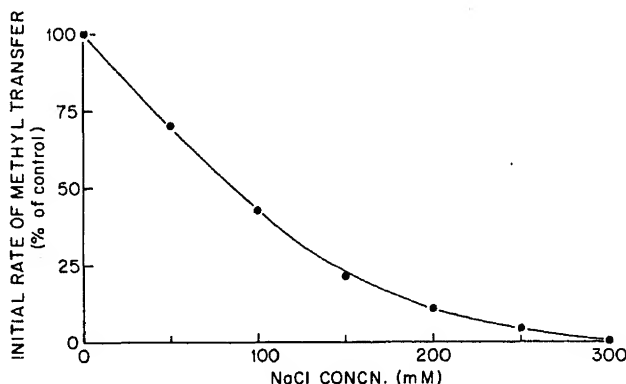


**Figure 1.** Kinetics of methyltransferase reaction at 37°. DNA substrate containing 160 fmol *O*<sup>6</sup>-methylguanine was incubated with either a 3 µl (○) or a 5 µl (●) aliquot of purified Fraction VII protein as described in 'materials and methods'. Duplicate samples were assayed for each time point. The inset shows the replot of data from the 5 µl reaction according to the second-order rate equation,  $kt = 1/(a-b) \ln b(a-x)/(a(b-x))$  (Frost and Pearson, 1961), where  $a$  equals the initial concentration of *O*<sup>6</sup>-methylguanine (160 fmol/200 µl =  $8.0 \times 10^{-10}$  M),  $b$  equals the initial concentration of methyltransferase ( $\sim 105$  fmol/200 µl =  $5.25 \times 10^{-10}$  M) based on the plateau level of [<sup>3</sup>H]-methyl protein, and  $x$  equals the amount reacted at time  $t$ .



**Figure 2.** Dependence of methyltransferase reaction rate on pH. One hundred fmol aliquots of methyltransferase were incubated with DNA containing 160 fmol *O*<sup>6</sup>-methylguanine as described in 'materials and methods' except that 20 mM K-PO<sub>4</sub> was replaced by other buffers at 20 mM adjusted to different pH values as follows: Na citrate:

Figure 3 shows that the initial rate of the methyltransferase reaction is very strongly dependent on NaCl concentration. This is surprising because even in buffer of physiological ionic strength of about 0.15 M, only about 25% of the control activity was observed. In confirmation of earlier studies with *E. coli* and rodent proteins, we did not observe any cofactor requirement for the human methyltransferase. Low concentrations of  $\text{MgCl}_2$ , EDTA and spermidine do not alter the initial reaction rate of the protein. However, 10 mM spermidine, 5 mM  $\text{AlCl}_3$  and 5 mM  $\text{FeCl}_2$  individually abolish the activity nearly completely (table 2). It is possible that the high concentrations of polyvalent ions either bind the methyltransferase molecules or alter the conformation of the DNA substrate to make  $O^6$ -methylguanine inaccessible.



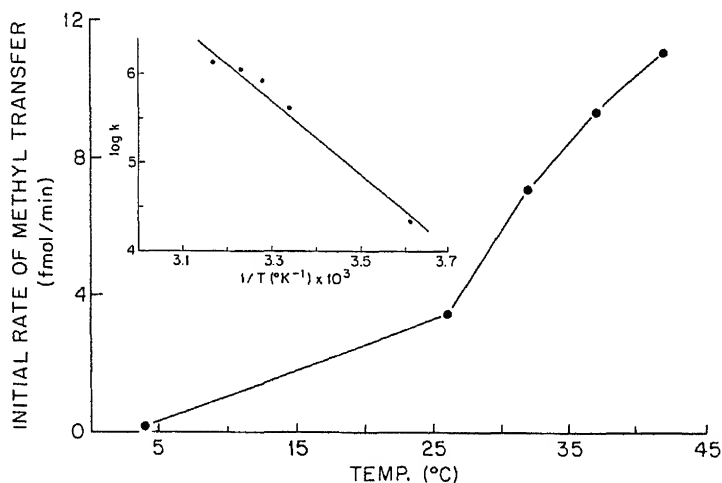
**Figure 3.** Effect of NaCl on methyltransferase reaction. In the presence of various NaCl concentrations, 100 fmol methyltransferase was incubated with the substrate and the activity measured as described in 'materials and methods'.

**Table 2.** Effect of polyvalent ions on methyltransferase activity (initial rate of reaction).

Addition	Activity (% of control)
*5 mM $\text{MgCl}_2$	76
1 mM $\text{MnCl}_2$	70
5 mM $\text{AlCl}_3$	6.9
5 mM $\text{FeCl}_2$	< 1
0.1 mM spermidine	100
1 mM spermidine	84
10 mM spermidine	< 1
No EDTA	79
0.1 mM EDTA	(100)
1.0 mM EDTA	87
10 mM EDTA	82



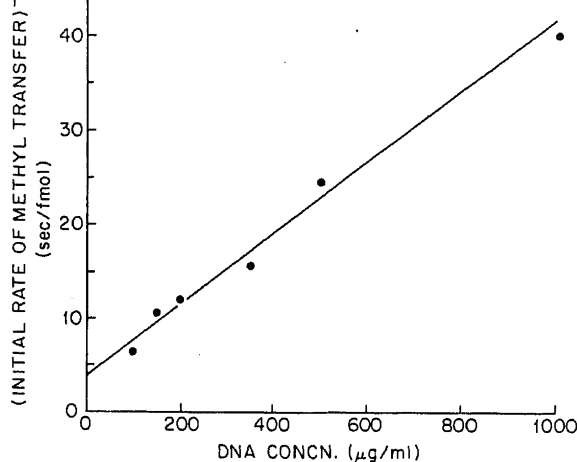
Figure 4 shows the effect of temperature on the initial rate of methyl-transfer reaction. The rate goes up continuously with temperature up to 42°C. In a separate experiment (not shown) we determined that the methyltransferase can be protected from heat inactivation at 50°C by the presence of duplex DNA. The inset in figure 4 shows the Arrhenius plot of the data; the energy of activation is calculated to be about 18 Cal. A comparison of the activation energy for the reaction of *O*<sup>6</sup>-methylguanine methyltransferase with other *O*<sup>6</sup>-alkylguanines may provide a basis for explaining the large differences in relative rates of dealkylation of *O*<sup>6</sup>-methyl-, -ethyl, and -isopropylguanine in DNA by the protein (Morimoto *et al.*, 1985).



**Figure 4.** Effect of temperature on the reaction rate of methyltransferase. The initial rate of reaction was determined at various temperatures. The inset shows the Arrhenius plot of the data.

### *Inhibition of methyltransferase reaction by DNA*

The *in vivo* rate of repair of *O*<sup>6</sup>-methylguanine in DNA obviously depends not only on the number of methyltransferase molecules in a cell, but also on the accessibility of the lesion to the protein. Because the mammalian DNA is present in a highly organized chromatin structure and there is a vast excess of DNA compared to the few lesions, we investigated whether excess DNA affects the methyltransfer reaction. Figure 5 shows that the initial rate of the reaction is inversely related to the concentration of duplex DNA. It is unlikely that increased viscosity is responsible for this inhibition because denatured DNA, at the same concentration, though much less viscous, is twice as inhibitory as duplex DNA. Thus, it appears that competitive non-specific binding of the methyltransferase to DNA in the low ionic strength of the reaction mixture may reduce the rate of formation of the complex of protein and *O*<sup>6</sup>-methylguanine.



**Figure 5.** Effect of DNA on methyltransferase. The initial reaction rate of the enzyme was measured as a function of DNA concentration which was increased by the addition of untreated duplex DNA.

In conclusion, we have shown that partially purified DNA-*O*<sup>6</sup>-methylguanine methyltransferase from human tissue is similar in its properties to the rodent methyltransferases. The initial rate of the methyltransferase reaction can be determined only by extrapolation to zero DNA concentration because of its inhibition by DNA. Further studies are necessary to determine whether the presence of histones and nonhistone chromosomal proteins affect the reaction rate. The strong inhibition of the reaction rate by salt is also surprising in view of the intracellular ionic strength. However, the inhibitory effects of excess DNA and physiological ionic strength can explain why the *in vivo* repair of *O*<sup>6</sup>-methylguanine (Pegg *et al.*, 1984) is significantly slower than the rate of repair observed with the purified enzyme.

## Acknowledgements

This research is supported by a USPHS grant CA 31721 and the Office of Health and Environmental Research, US Department of Energy under contract DE-ACO5-84OR21400 with the Martin Marietta Energy Systems, Inc. The authors would like to thank Mr. W. C. Dunn for experimental assistance and to the Oak Ridge Methodist Medical Center and the University of Tennessee Memorial Research Center for providing human placentas.

## References

- Bogden, J. M., Eastman, A. and Bresnick, E. (1981) *Nucleic Acids Res.*, **9**, 3089.
- Cleaver, J. E. and Kaufmann, W. K. (1980) *Nature (London)*, **284**, 378.
- Dolan, M. E., Scicchitano, D., Singer, B. and Pegg, A. E. (1984) *Biochem. Biophys. Res. Commun.*, **23**, 324.
- Foote, R. S. and Mitra, S. (1984) *Carcinogenesis*, **5**, 277.

- Frost, A. A. and Pearson, R. G. (1961) *Kinetics and Mechanism: A Study of Homogeneous Chemical Reactions*, 2nd edition (New York: John Wiley and Sons) p. 15.
- Goth, R. and Rajewsky, M. F. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 639.
- Hora, J. F., Eastman, A. and Bresnick, E. (1983) *Biochemistry*, **22**, 3759.
- Jeggo, P. (1979) *J. Bacteriol.*, **139**, 783.
- Jeggo, P., Defais, M., Samson, L. and Schendel, P. (1977) *Mol. Gen. Genet.*, **157**, 1.
- Karran, P., Arlett, C. F. and Broughton, B. C. (1982) *Biochemie*, **64**, 717.
- Karran, P., Lindahl, T. and Griffin, B. (1979) *Nature (London)*, **280**, 76.
- Lindahl, T., Demple, B. and Robins, P. (1982) *EMBO J.*, **1**, 1359.
- Loveless, A. (1969) *Nature (London)*, **223**, 206.
- McCarthy, T. V., Karran, P. and Lindahl, T. (1984) *EMBO J.*, **3**, 545.
- McCarthy, T. V. and Lindahl, T. (1985) *Nucleic Acids Res.*, **13**, 2683.
- Marigson, G. P., Cooker, D. P. and Brennand, J. (1985) *Nucleic Acids Res.*, **13**, 1939.
- Mehta, J., Ludlum, D. B., Renard, A. and Verly, W. G. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6766.
- Mitra, S., Pal, B. C. and Foote, R. S. (1982) *J. Bacteriol.*, **152**, 534.
- Montesano, R. H., Margison, G. P. and Bresil, G. P. (1979) *Cancer Res.*, **39**, 1789.
- Myrnes, B., Giercksky, K. E. and Krokan, H. (1982) *J. Cell Biochem.*, **20**, 381.
- Morimoto, K., Dolan, M. E., Scicchitano, D. and Pegg, A. E. (1985) *Carcinogenesis*, **6**, 1027.
- Nicoll, J. W., Swann, P. F. and Pegg, A. E. (1975) *Nature (London)*, **254**, 261.
- Olsson, M. and Lindahl, T. (1980) *J. Biol. Chem.*, **255**, 10569.
- Pegg, A. E., Robefroid, M., vonBahr, C., Foote, R. S., Mitra, S., Bresil, H., Likhachev, A. and Montesano, R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5162.
- Pegg, A. E., Scicchitano, D. and Dolan, M. E. (1984) *Cancer Res.*, **48**, 3806.
- Pegg, A. E., Wiest, L., Foote, R. S., Mitra, S. and Perry, W. (1983) *J. Biol. Chem.*, **258**, 2327.
- Potuzak, H. and Wintersberger, U. (1976) *FEBS Lett.*, **63**, 167.
- Robins, P. and Cairns, J. (1979) *Nature (London)*, **280**, 74.
- Samson, L. and Cairns, J. (1977) *Nature (London)*, **267**, 281.
- Schendel, P. and Robins, P. E. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 6017.
- Sklar, R. and Strauss, B. (1981) *Carcinogenesis*, **2**, 1293.
- Snow, E. T., Foote, R. S. and Mitra, S. (1984) *J. Biol. Chem.*, **259**, 8095.
- Sober, H. A. (1968) in *Handbook of Biochemistry*, (Cleveland: The Chemical Bubber Co.) p. J-81.
- Teo, I. Sedwick, B., Demple, B., Lu, B. and Lindahl, T. (1984) *EMBO J.*, **3**, 2151.
- Teo, I. Sedwick, B., Kilpatrick, M. W., McCarthy, T. W. and Lindahl, T. (1986) *Cell*, **45**, 315.
- Topal, M. D., Eadie, J. S. and Conrad, M. (1986) *J. Biol. Chem.*, **261**, 9879.
- Waldstein, E. A., Cao, E. H. and Setlow, R. B. (1982) *Nucleic Acids Res.*, **10**, 4595.
- Yarosh, D. B., Rice, M., Day, R. S., Foote, R. S. and Mitra, S. (1984) *Mutat. Res.*, **131**, 27.
- Yarosh, D. B., Furnace, A. J. and Day, R. S. (1985) *Carcinogenesis*, **6**, 949.

ROBERTA HABER and SANKAR ADHYA

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 USA

**Abstract.** Autoregulation of the *rho* gene insures a controlled level of a critical gene product independent of cellular changes. We have investigated the autoregulation of *rho*, the gene that encodes the transcription termination factor, *rho*. In a DNA dependent *in vitro* coupled transcription-translation system, *rho* represses its own synthesis, confirming the autoregulatory nature of *rho*. *rho* is believed to perform its negative regulatory role by modulating transcription termination at an early site in the operon.

**Keywords.** Autoregulation; *rho* gene; transcription termination.

## Introduction

The essential feature of autoregulation is the direct modulation of gene expression by the product of the same gene. Thus, a protein controls the rate of synthesis of additional copies of itself.

Autoregulation is not a new concept in genetics; the phenomenon has been known in prokaryotic and eukaryotic organisms for more than two decades. However, autoregulation is newly appreciated as a mechanism of regulation of genes whose products perform critical cellular functions. Autoregulation provides a mechanism for amplification of gene expression (Kourilsky and Gros, 1974), for regulation of proteins involved in DNA replication (Braun *et al.*, 1985), for buffering the response of genes to environmental changes (McFall and Bloom, 1971; Hagen and Magasanik, 1973), and for maintaining a constant intracellular concentration of a gene product, independent of cell size, cell growth rate and changes in gene copy number (Sompayrac and Maaløe, 1973).

A genetic mechanism which is not autoregulatory may appear so on the surface. For example, a gene or operon whose product(s) participate in the synthesis of the gene's inducer or co-repressor could appear to be an autoregulated system, if the biochemistry were not known. The best way to demonstrate the existence of an autoregulatory system and to discern the molecular mechanism of that control is to duplicate the control system *in vitro*.

The expression of many of the global control genes of *Escherichia coli* appears to be self-regulated, allowing a critical level of the gene product to be maintained in the cell. An example of one such gene is that of the transcription termination protein, *rho*. Ratner first observed that in *rho*<sup>-</sup> mutant *E. coli* cells, the defective *rho* protein was present at a higher level than *rho* protein in wild type cells (Ratner, 1976). He suggested that the *rho* gene is autoregulatory and *rho* protein controls its level by modulating transcription termination at the beginning of the *rho* gene. The enhanced expression of the *rho* gene in the mutant cells has been confirmed *in vivo* in two ways: (i) In *rho*<sup>-</sup> mutant backgrounds there is enhanced  $\beta$ -galactosidase synthesis from a chromosomal *rho-lacZ* protein fusion (Garges, 1983) and enhanced galactokinase synthesis from a *rho-galK* operon fusion plasmid (Barik *et al.*, 1985).

(ii) The rate of synthesis (Brown *et al.*, 1982) as well as the steady state level (Garges, 1983; Barik *et al.*, 1985) of *rho* mRNA is higher in *rho*<sup>-</sup> cells than in *rho*<sup>+</sup> cells. These results argue in favor of an autoregulated *rho* gene.

In this paper, we examine autoregulation of *rho* in a coupled transcription-translation system and provide evidence that the *rho*112 mutant is altered in autoregulation.

## Materials and methods

### Strains

The list of *E. coli* K-12 strains and plasmids used in this study is presented in table 1.

**Table 1.** Strains and plasmids used in this study.

Strain	Genotype	Reference
<i>E. coli</i> K12:		
G737	F <sup>-</sup> <i>su</i> <sup>-</sup> <i>recB21 recC22 sbcB15</i> <i>Thr1 leuB6 Thi1 lacY1 galK2</i> <i>ara14 xyl5 mtl1 proA2 his4</i> <i>argE3 rpsL31 tsx33</i>	(Garges, 1983)
G736	G737 <i>rho</i> 112*	(Garges, 1983)
Plasmid:		
pBR322	<i>bla</i> <sup>+</sup> <i>tet</i> <sup>+</sup>	(Sutcliffe, 1979)
pEG25	<i>bla</i> <sup>+</sup> <i>rho</i> <sup>+</sup>	(Gulletta <i>et al.</i> 1985)

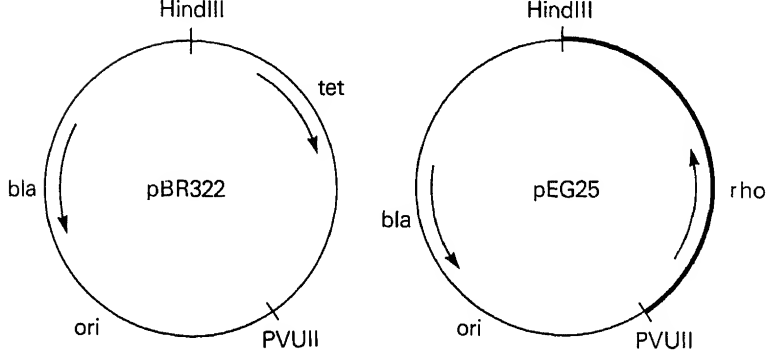
\**rho*112 mutation is described in Das *et al.* (1978).

### Coupled transcription-translation reactions

S-30 extracts were prepared by Codon Co. of Houston, Texas, USA according to the method of Zubay (1973). Components of the reaction were 20 mM Tris-acetate pH 8.0, 8 mM KOAC, 0.01 mM of all amino acids except methionine, 2 mM adenosine 5'-triphosphate (ATP), 0.5 mM UTP, 0.5 mM GTP, 0.5 mM CTP, 0.2 mg/ml *E. coli* tRNA, 10 µg/ml pyruvate kinase, and 5 mM phosphoenolpyruvate. Each 10 µl reaction contained 0.25 µg cesium chloride banded plasmid DNA and 2.5 µCi [<sup>35</sup>S]-methionine (Amersham, 600 Ci/mMol). Reactions were carried out at 37° for 40 min, then stopped by addition of trichloroacetic acid (TCA) to a final concentration of 10%. Samples were precipitated, washed with cold acetone and suspended in sample buffer for sodium dodecyl sulphate (SDS)/polyacrylamide gels. 10% polyacrylamide gels were run according to the method of Laemmli (1970).

## Results

S-30 extracts for coupled transcription-translation reactions were made from wild type *E. coli* (G737) and from a *rho*112 mutant strain (G736) as described in 'materials and methods'. These extracts were used to carry out protein synthesis directed by plasmid pEG25. pEG25 is a derivative of pBR322 carrying a 2.9 kbp DNA fragment

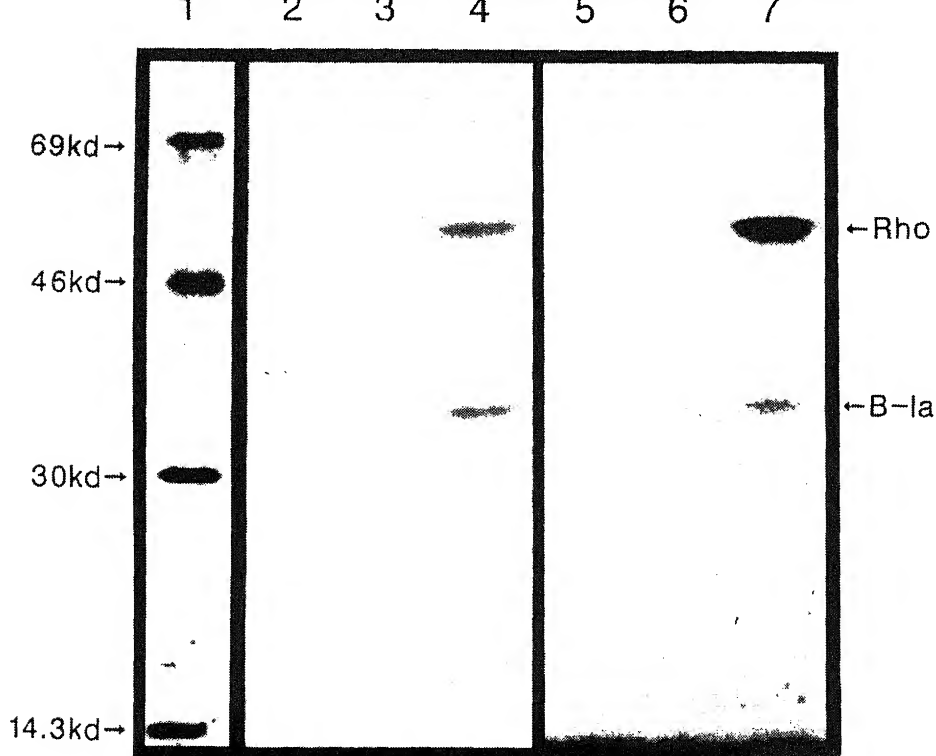


**Figure 1.** Diagrammatic representation of vector plasmid pBR322 (left) and  $\rho^+$  plasmid pEG25 (right). The bacterial DNA containing the  $\rho^+$  gene is shown by the thick line. The construction is described in Gulletta *et al.* (1985). The arrows represent direction of transcription. *bla*, gene for Ampicillin resistance; *tet*, gene for tetracyclin resistance;  $\rho$ , structural gene for  $\rho$ .

vector (Gulletta *et al.*, 1985). The structure of pEG25 is shown in figure 1. Protein synthesis was carried out in the presence of [ $^{35}$ S]-methionine as described by Zubay (1973). The incubation mixture was analyzed for labelled proteins by SDS-polyacrylamide gel electrophoresis (PAGE). An autoradiograph of the results of this coupled transcription-translation reaction is shown (figure 2). S-30 extracts with no added DNA do not show any labelled protein, confirming the absence of endogeneous DNA (figure 2, lanes 2 and 5). In the presence of both pBR322 and pEG25, a 33 kd protein is made in both the  $\rho^+$  S-30 and the  $\rho$ 112 mutant S-30 (figure 2, lanes 3 and 6). The 33 kd band is the precursor of  $\beta$ -lactamase directed by the *bla* gene present on both plasmids. pEG25 directs the synthesis of an additional protein whose mobility corresponds to a molecular weight of 50 kd (figure 2, lanes 4 and 7). We conclude that this protein is  $\rho$  monomer because its estimated size is consistent with the size of  $\rho$  deduced from the DNA sequence of the  $\rho$  gene (Pinkham and Platt, 1983), and because the radioactive band comigrates with purified  $\rho$  in SDS-PAGE (data not shown).

These results demonstrate DNA-dependent synthesis of  $\rho$  protein *in vitro* using crude cell-free extracts made both from  $\rho^+$  and  $\rho$ 112 strains as a source of the components of the transcription-translation reaction.

We have scanned the intensity of various protein bands in the autoradiograms to determine the relative amounts of  $\rho$  and  $\beta$ -lactamase synthesized. The quantitation of the scanning is shown in table 2. The results are expressed assuming the amount of  $\beta$ -lactamase monomers synthesized under the conditions described in 'materials and methods' as unit in each extract. Compared to the synthesis of  $\beta$ -lactamase, the synthesis of  $\rho$  is 7-fold higher in the  $\rho$ 112 extract than in the  $\rho^+$  extract. The differential ability of wild type and mutant extracts is similar to that observed *in vivo* as discussed above. These results show the following: If we assume that protein stability is the same in both extracts during the 40 min labelling period, our results confirm that the difference in  $\rho$  levels between  $\rho^+$  and  $\rho^-$  backgrounds is not at the level of activity, but at the level of synthesis. In addition,



**Figure 2.** Coupled transcription-translation reactions were carried out and products run on 10% SDS-polyacrylamide gels, as described in 'materials and methods'. Lane 1, molecular weight markers; lanes 2-4, wild-type extracts; lanes 5-7, *rho112* extract. The reactions shown in lanes 2 and 5 contain no exogenous DNA; the reactions shown in lanes 3 and 6 contain 0.25 µg of pBR322; the reactions shown in lanes 4 and 7 contain 0.25 µg of pEG25.

**Table 2.** DNA directed synthesis of proteins *in vitro*.

Extract	Relevant genotype	<i>B</i> -lactamase	<i>rho</i>
G737	<i>rho</i> <sup>+</sup>	1	1.1
G736	<i>rho112</i>	1	7.5

of the two extracts is the presence and absence of active *rho* protein, we conclude that *rho* protein inhibits its own synthesis. The implications of these conclusions are discussed next.

## Discussion

Many genes are autoregulated. In an autoregulated system, the product of a gene directly participates in regulating the expression of that gene. The best evidence that

tract is capable of increased *de novo* synthesis of *rho* protein indicates that *rho* is autoregulated *in vitro*. Results similar to ours have been obtained independently by Ung *et al.* (1984) and Barik *et al.* (1985) using other *rho*<sup>-</sup> mutants. These authors have also shown that inactivation of *rho* protein in a wild type extract by addition of anti-*rho* antibody increased plasmid-directed *rho* synthesis, while addition of purified *rho* protein to a *rho*<sup>-</sup> extract decreased *rho* synthesis to the wild type level. *Rho* protein is an essential regulator of transcription in *E. coli* (Das *et al.*, 1976; Adhya and Gottesman, 1978). The ability of *rho* protein to repress *rho* gene expression provides an efficient mechanism to maintain a constant intracellular level of *rho*, independent of growth rate and other cellular changes. Plasmid-directed *rho* gene expression is higher in the *rho*112 extract than in the wild-type extract. When the *rho* protein is defective, the cell may need more of it than it would need of wild-type *rho* in order to fulfill its termination requirements. Therefore, an apparent defect in autoregulation may actually save the *rho*112 mutant strain by providing the cell with higher levels of the inefficient protein. This ability to compensate for a defective *rho* protein by overproduction illustrates a benefit of autoregulation.

How does *rho* negatively control gene expression? *rho* is a factor in transcription termination. Termination of RNA synthesis in *E. coli* occurs at specific sites on the genome, resulting in functional RNAs of discrete sizes (Adhya and Gottesman, 1978). At many such sites, the protein factor *rho* is required for RNA polymerase to halt activity, thus preventing transcription of promoter distal genes (Roberts, 1969; de Crombrughe *et al.*, 1973).

*Rho* protein is a hexamer composed of identical 48 kilodalton subunits which assume a ring-like conformation (Galluppi and Richardson, 1980). Two regions of the *rho* protein have the capacity to bind RNA *in vitro*. The primary binding region of the protein binds to a region of RNA which is approximately 60 bases long. Concurrently, the secondary binding region binds an 8-base region of RNA. The secondary binding is accompanied by hydrolysis of ATP (Richardson and Corey, 1982).

Basically, there are 3 steps for *rho*-dependent termination of transcription (Adhya and Gottesman, 1978; Das *et al.*, 1978; Morgan *et al.*, 1983, 1984). The first event is the *rho*-independent pausing of RNA polymerase at specific termination sites on the DNA template. The second event is the binding of *rho* protein to a long stretch of RNA (approximately 70-90 nucleotides) which has minimal secondary structure. The last step is *rho* binding to the paused RNA polymerase, coupled with ATP hydrolysis and release of polymerase and RNA from the DNA template. The NusA protein of *E. coli*, which has been shown to bind both *rho* and RNA polymerase *in vitro*, may mediate the *rho*-driven separation of RNA polymerase from the template (Schmidt and Chamberlin, 1984).

A number of *rho* mutants have been isolated and demonstrated to have a defect at one of these steps of transcription termination. As we have discussed, these *rho* mutants are defective in autoregulation and show increased *rho* transcription, strongly suggesting that *rho* modulates its own synthesis at the level of transcription termination. In fact, Brown *et al.* (1982) and Matsumoto *et al.* (1986) identified by S1 nuclease analysis of *rho* mRNA several intraoperonic termination sites preceding the *rho* coding region. Brown *et al.* (1982) suggested that *rho* may be modulating *rho* mRNA degradation at these sites. We are currently analyzing the sites of *rho* action both



## Acknowledgement

We thank Susan Garges for many helpful discussions and strains.

## References

- Adhya, S. and Gottesman, M. (1978) *Annu. Rev. Biochem.*, **47**, 967.
- Barik, S., Bhattacharya, P. and Das, A. (1985) *J. Mol. Biol.*, **182**, 495.
- Braun, R. E., O'Day, K. and Wright, A. (1985) *Cell*, **40**, 159.
- Brown, S., Albrechtsen, B., Pederson, S. and Klem, P. (1982) *J. Mol. Biol.*, **162**, 283.
- Das, A., Court, D. and Adhya, S. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 1959.
- Das, A., Merrill, C. and Adhya, S. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4828.
- de Crombrughe, B., Adhya, S., Gottesman, M. and Pastan, I. (1973) *Nature (London)*, **241**, 260.
- Galluppi, G. R. and Richardson, J. P. (1980) *J. Mol. Biol.*, **138**, 513.
- Garges, S. (1983) The regulation of the *rho*, *crp*, and *cya* genes of *Escherichia coli*, Ph.D. Thesis, University of Maryland, College Park, Maryland, USA.
- Gulletta, E., Spagnuolo, G. and Adhya, S. (1985) *Microbiologia*, **8**, 303.
- Hagen D. C. and Magasanik, B. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 808.
- Kourilsky, P. and Gros, F. (1974) in *Regulation of Gene Expression in Cultured Cells*. Fogarty International Conference Center Proceedings, No. 22, US Government Printing Office, Washington, D.C.
- Kung, H. F., Bekesi, E., Guterman, S. K., Gray, J. E., Traub, L. and Calhoun, D. H. (1984) *Mol. Genet.*, **193**, 210.
- Laemmli, U. K. (1970) *Nature (London)*, **227**, 680.
- Matsumoto, Y., Shigesada, K., Hirano, M., and Imai, M. (1986) *J. Bacteriol.*, **166**, 945.
- McFall, E. and Bloom, F. R. (1971) *J. Bacteriol.*, **105**, 241.
- Morgan, W. D., Bear, D. G. and von Hippel, P. H. (1983) *J. Biol. Chem.*, **258**, 9565.
- Morgan, W. D., Bear, D. G. and von Hippel, P. H. (1984) *J. Biol. Chem.*, **259**, 8664.
- Pinkham, J. L. and Platt, T. (1983) *Nucl. Acid Res.*, **11**, 353.
- Ratner, D. (1976) *Nature (London)*, **259**, 151.
- Richardson, J. P. and Corey, J. L. (1982) *J. Biol. Chem.*, **257**, 5767.
- Roberts, J. (1969) *Nature (London)*, **224**, 1168.
- Schmidt, M. C. and Chamberlin, M. J. (1984) *J. Biol. Chem.*, **259**, 15000.
- Sompayrac, L. and Maaløe, O. (1973) *Nature New Biol.*, **241**, 133.
- Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 77.
- Zubay, G. (1973) *Annu. Rev. Genet.*, **7**, 267.

# Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of *Vibrio cholerae*

UMA BHATTACHARYYA\*, AMIT GHOSH and  
RANAJIT K. GHOSH

Indian Institute of Chemical Biology, Jadavpur, Calcutta 700 032, India

\*Present Address: Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514, USA

**Abstract.** Non-pathogenic, environmental strain of *Vibrio cholerae*, ELTOR Ogawa EW6 carries a copy of the cholera toxin gene in its chromosome. Restriction enzyme digestion followed by Southern blot analysis revealed that the structure of the cholera toxin gene in this organism is different from that found in the virulent strains. The *xbal* site which has been found to be conserved in the cholera toxin of the virulent strains examined so far, is absent here. Results of the RNA dot blot analysis indicated that the cholera toxin gene in EW6 is transcribed much less efficiently compared to the cholera toxin gene present in the virulent strain *Vibrio cholerae* classical Inaba 569B.

**Keywords.** *Vibrio cholerae*; avirulent strain; toxin gene.

## Introduction

The role of non-pathogenic strains of *Vibrio cholerae* found in the aquatic environment, is an intriguing question in the epidemiology of the disease cholera. Each year cholera epidemics surface simultaneously in many places in different third world countries. It is widely believed that these epidemics spring *de novo* from aquatic environments. Very little is known about these environmental isolates, excepting that they are unable to cause infection in man and that their non-pathogenic character is quite stable. Attempts have been made to use some of these isolates as live oral vaccine for cholera without much success (Cash *et al.*, 1974; Levine *et al.*, 1982). Recently there has been a resurgence of interest in these strains and it has been found that while the majority of the non-pathogenic, environmental strains lack the structural gene coding for the cholera toxin (*ctx*) (Kaper *et al.*, 1981), there are a few strains including some non-01 vibrios which carry copies of toxin gene in their chromosomes (Hanchalay *et al.*, 1985). However, nothing is known about the organization of the toxin gene in the avirulent strains or its expression there.

We report here our work on the non-pathogenic environmental strain *V. cholerae* ELTOR Ogawa EW6 (hereafter designated as EW6), isolated by Dr. S. Mukherjee from a water source in 1958 (Cash *et al.*, 1974). We show that this strain though unable to elaborate any toxin as measured by rabbit ileal loop test or vascular permeability test, even after repeated propagation (Gerdes and Romig, 1975; present work), nevertheless carries a copy of the toxin gene in its chromosome. The structure of the gene however is different from that found in the virulent strains. We further show that compared to the *ctx* present in the pathogenic strain *V. cholerae* classical Inaba 569B (henceforth designated as 569B), the *ctx* in EW6 is transcribed with much lower efficiency.

The strains used in this study *viz.* non-pathogenic *V. cholerae* ELTOR Ogawa EW6 (Cash *et al.*, 1974) and the pathogenic strains *V. cholerae* classical Inaba 569B and *V. cholerae* classical Ogawa 154 (Mukherjee 1978) were obtained from Dr. F. K. Bhattacharyya of our Institute. Cultures were maintained under conditions described by Mitra *et al.* (1986).

### *Media and growth conditions*

Cells were grown as described before (Siddiqui and Ghosh, 1983) in nutrient broth containing Bactopeptone (Difco) 10 g, Lab-lemco powder (Oxoid) 10 g, Sodium chloride 5 g, per litre of glass distilled water. The pH of the medium was adjusted to 8.0. Nutrient agar plates contained 1.5% (w/v) Bactoagar (Difco) in nutrient broth.

### *Colony Hybridization*

This was done essentially according to the method of Grunstein and Hogness (1975). Bacterial colonies from the master plate was transferred to a nitrocellulose disc (Schleicher and Schuell, Keene, N. H., USA). Colonies were lysed *in situ* and the DNA imprint was fixed to the disc by baking. Hybridization to [<sup>32</sup>P]-labelled cholera toxin gene probe was done as described below. After hybridization the filter was monitored by autoradiography.

### *Toxin assay*

Either whole cell lysates or cell free culture supernatants were used as toxin preparations. Lysate preparation by sonication was essentially according to Burrows *et al.* (1965). The amount of toxin produced by a culture was assayed by (i) the rabbit ileal loop method (Kasai and Burrows, 1966); (ii) the vascular permeability test (Craig, 1971). In rabbit ileal loop test, not more than 100 cm of gut measuring from anterior to appendix was used. Two ml of cell lysates or cell free supernatants was inoculated directly or after suitable dilutions, in to five 10–12 cm loops. Negative (broth) and positive (animal passaged 569B cells) control loops were included in all experiments. Fluid accumulation in ligated loops, after 24 h was taken as a measure of toxicity. Amount of toxin produced was expressed as the ratio of the volume of fluid accumulated to the length of the negative control loop.

In vascular permeability test 0.1 ml of the suitable dilutions of the cell-free culture supernatant was injected intradermally on the depilated back of rabbits. After 24 h, a 5% (w/v) solution of pontamine sky blue 6Bx (Gurr, England) was injected intravenously (1.2 ml per kg body weight). The diameter of the blue lesion was measured 1 h after the injection of the dye. Toxin level was expressed as the blueing dose per ml of culture. One blueing dose is equivalent to a blue lesion of 7 mm diameter (Craig, 1971).

then fractionated by electrophoresis in vertical 1% Agarose gels containing 40 mM Tris (pH 8.3), 20 mM Sodium acetate and 1 mM EDTA. The fragments were denatured and then transferred to nitrocellulose sheets (Schleicher and Scuell, Keene, N. H., USA) by the method of Southern (1975). The highly purified cholera toxin gene probe (kindly provided by Dr. P. J. Greenaway) was labelled with [ $^{32}$ P]-by nick translation (Rigby *et al.*, 1977). Hybridization solution consisted of buffer containing 50% Formamide, 5  $\times$  SSC, 1 mM EDTA, 1% sodium dodecyl sulphate (SDS), 100  $\mu$ g/ml sheared calf thymus DNA and 1  $\times$  Denhardt's solution (Denhardt, 1966). Hybridization was performed at 37°C for 24–36 h in a sealed plastic bag. Blots were first washed in 5  $\times$  standard saline citrate (SSC) – 0.1% SDS for 45 min at 55°C and then in 2  $\times$  SSC for 15 min at room temperature. These were then autoradiographed for 3–7 days at –70°C using intensifying screens.

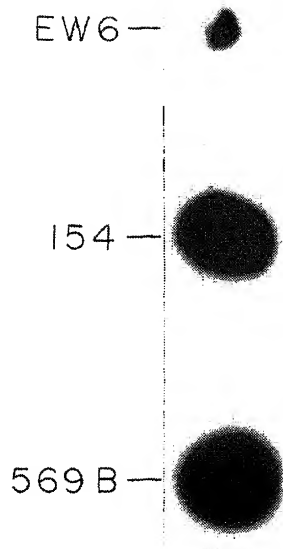
### *RNA blot analysis*

RNA for the RNA blot hybridization was purified according to Aiba *et al.* (1981), from log phase cells of *V. cholerae*, growing in CYE medium (Mekalanos *et al.*, 1977). 100  $\mu$ g of purified RNA was dissolved in 400  $\mu$ l of sterile glass distilled water. To this 1.2 ml of a solution of 6.15 M formaldehyde, 10  $\times$  SSC was added. The resulting mixture was heated to 65°C for 15 min and then transferred to a Nitrocellulose sheet using a Minifold II slot blot system (Schleicher and Schuell, Keene, N. H., USA), according to the method recommended by the manufacturer. Hybridization with [ $^{32}$ P]-labelled cholera toxin probe was done under reduced stringency condition (25% formamide) according to Thomas (1983).

## **Results**

### *Examination for the presence of toxin gene (ctx) in V. cholerae EW6*

*In situ* colony hybridization method of Grunstein and Hogness (1975) was used to screen *V. cholerae* EW6 for the presence of DNA sequences homologous to the *ctx* gene. Bacterial colonies were grown on nitrocellulose filter, denatured *in situ* and then hybridized with [ $^{32}$ P]-labelled cholera toxin gene probe. As can be seen from the autoradiograph (figure 1), EW6 gave a positive signal indicating that a *ctx* like sequence is present in it. Closer examination of the autoradiograph revealed that while both the virulent strains gave strong positive reaction, the hybridization signal obtained with EW6 was somewhat weaker. Since the hybridization was carried out under highly stringent condition (50% formamide), it indicated that either the *ctx* sequence present in EW6 is closely homologous but probably not identical to those found in the toxigenic strains or that only a single copy of *ctx* is present in EW6, while the virulent strains carry more than one copy of the *ctx* gene.



**Figure 1.** Colony-hybridization of pathogenic and non-pathogenic strains of *V. cholerae* with CT gene probe.

#### *Enterotoxinogenicity of EW6 after animal passage*

It has been reported in the literature that the non-pathogenic character of the environmental strain EW6 is quite stable (Gerdes and Romig 1975). In view of our observation that the strain EW6 carries a sequence closely homologous to the *ctx* gene. We thought it necessary to reconfirm this observation. The result of our experiment is summarised in table 1. As can be seen, no detectable amount of toxin was produced in EW6. In contrast the strain 569B exhibited a marked increase in toxin production.

**Table 1.** Toxin production by *V. cholerae* strains before and after animal passage.

Status	Toxin production measured			
	Fluid accumulation in ileal loop ml/cm		Blueing dose/ml	
	EW6	569B	EW6	569B
Before animal passage	0	0.48 ± 0.15	0	650 ± 55
After first animal passage	0	1.52 ± 0.25	0	1600 ± 125
After second animal passage	0	1.64 ± 0.20	0	2100 ± 75

Each value represents mean of 5 independent determinations.

#### *Structural characteristics of the ctx gene in EW6*

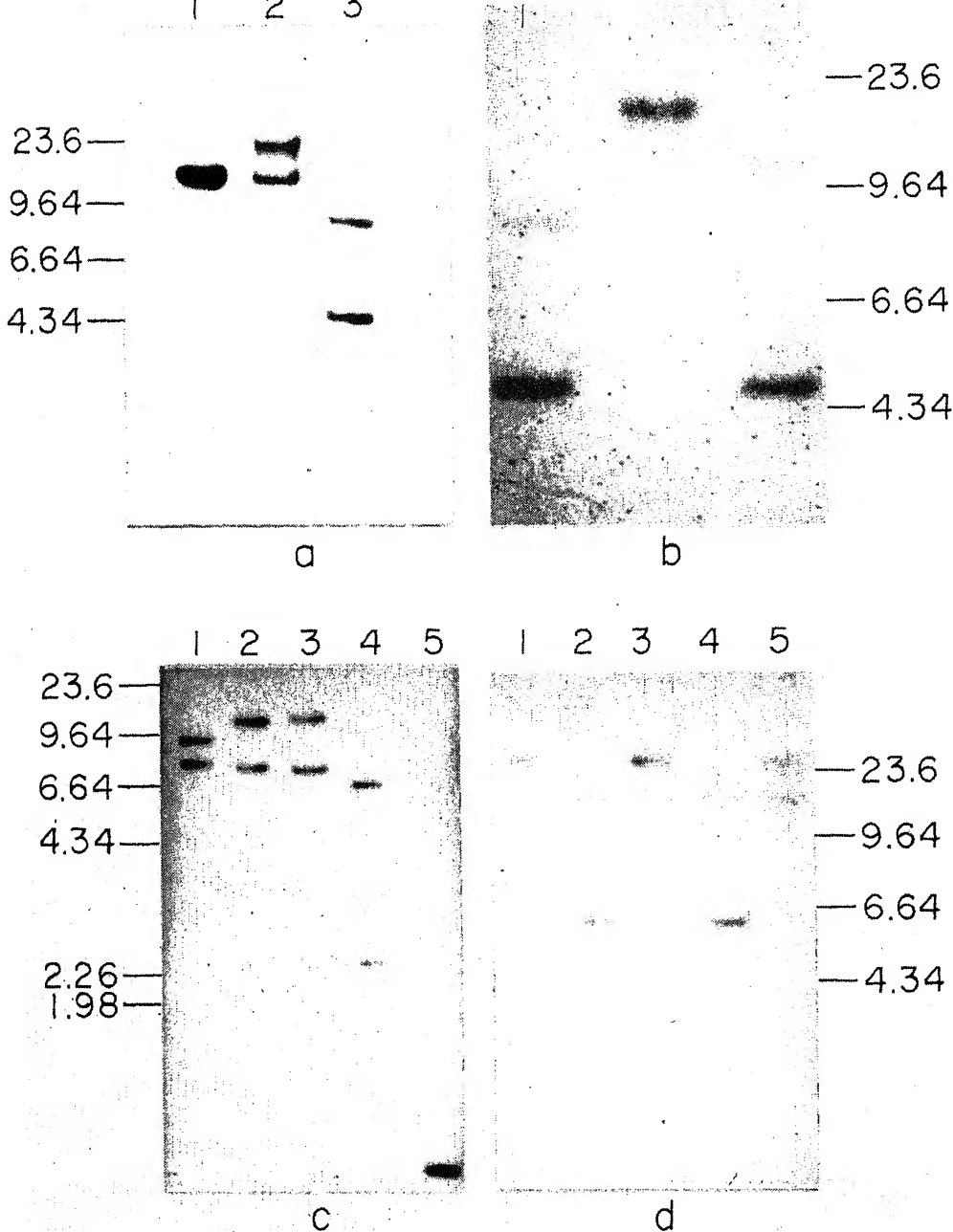
son (figure 2). Several interesting differences in the digestion pattern was observed. Strain 569B is known to carry a toxin gene duplication and yields two bands on Southern blot when analysed with *Xba* I, *Pst* I, *Bgl* II and *Eco*RI. *Xba* I site is known to be conserved among several cholera toxin genes derived from both classical and ELTOR strains (Gennaro *et al.*, 1982; Gennaro and Greenaway, 1983; Mekalanos *et al.*, 1983). Moreover in all ELTOR strains which contain only a single copy of the cholera toxin operon, *ctx* is found to be located on *Xba* I fragments of 1 kb or less (Mekalanos, 1983). In contrast, in EW6 it was found to be located on a fragment larger than 23 kb in size, indicating that in EW6, the *Xba*I site is not conserved. When the analysis of EW6 DNA was carried out with *Bgl* II, *Pst* I and *Hind* III in each case it produced two bands of unequal intensity on the Southern blot (figure 2). This was found to be a highly reproducible pattern and could not be an artefact. The only way to interpret this data it seems, is to assume that *ctx* in EW6 is not duplicated (for if it were then one would expect the two bands to be of equal intensity) but rather that these enzymes cleave EW6 *ctx* assymmetrically giving rise to one large fragment spanning most of the *ctx* and one small fragment, and it is this smaller fragment which gives rise to the weaker band on the autoradiograph. It is of interest to note here that all the *V. cholerae* ELTOR strains examined so far give rise to only one band on Southern blot when *Hind* III is used for analysis. Double digestion patterns also revealed interesting differences between the *ctx* of 569B and that of EW6. Digestion of 569B chromosomal DNA with *Xba*I and *Hinc* II is known to release a 1 kb fragment (figure 2, Mekalanos *et al.*, 1983) from the *ctx* gene. This however was not observed with the *ctx* present in EW6 (figure 2). It is worth mentioning here that *Xba* I + *Hinc* II digestion of chromosomal DNA of ELTOR 1621 a virulent strain and the only one ELTOR strain on which this particular double digestion analysis has been done also releases a 1 kb fragment (P. J. Greenaway, personal communication).

#### Quantitation of toxin gene specific mRNA by slot blot analysis

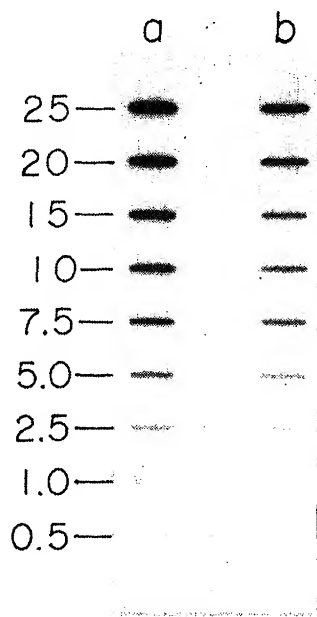
The presence of the toxin gene in EW6, though of a different structure, and the absence of toxin production as determined by rabbit ileal loop assay and vascular permeability test, prompted us to examine the expression of this gene *in vivo*. To quantitate the amount of toxin gene specific mRNA, if at all made, in this strain, we performed an RNA dot blot analysis as described in 'materials and methods'. It can be seen from figure 3 that a much lower level of *ctx* gene specific mRNA is made in *cholerae* EW6 compared to that in the virulent strain 569B.

#### Discussion

The data presented in this paper show that the environmental non-pathogenic strain *V. cholerae*, ELTOR Ogawa EW6, even though phenotypically non-toxinogenic in character, nonetheless carries a copy of the toxin gene in its chromosome. The *ctx* gene present in this organism, however, has a structure different from that found in its pathogenic cousins. RNA blot analysis revealed that in contrast to the *ctx* present in



**Figure 2.** Southern blot analysis of *V. cholerae* DNA derived from strain 569B (a and c) and EW6 (b and d) using [ $^{32}$ P]-labelled toxin gene probe. DNA (1  $\mu$ g) from each strain was digested with various enzymes singly or in combination, fractionated by electrophoresis on agarose gel, blotted and hybridized with nick translated CT gene probe. Lanes 1-3 (a and b) represent digestion with *Hind* III, *Eco*RI and *Pst* I. Lanes 1-5 (c and d) show digestion with *Xba* I, *Bgl* II, *Hinc* II, *Xba* I + *Bgl* II and *Xba* I + *Hinc* II, respectively. Positions of  $\lambda$  DNA/Hind III fragments (kb) run on the same gel are indicated by arrows.



**Figure 3.** Slot blot analysis of the toxin gene transcripts synthesized by *V. cholerae* strain 569B (a) and EW6 (b). Total RNA was isolated from both the strains as described in 'materials and methods'. This was then denatured and immobilized on nitrocellulose membranes. The filter was then hybridized with nick translated CT gene probe. The amount of RNA ( $\mu\text{g}$ ) loaded per slot in either case is indicated in the margin.

the virulent strain 569B, *ctx* in EW6 is transcribed with reduced efficiency. Why this is so is not clear at the moment. It could be because of some defect in the putative toxin regulatory gene (*tox R*) found to be present in all *V. cholerae* strains examined so far or it could be because of some aberration in the promoter region of the *ctx* itself (Miller and Mekalanos 1984). Another unsolved riddle is the apparent non-toxinogenic phenotype of EW6 despite the presence of *ctx* and its expression in it. It may be that the reduced amount of toxin it makes, is not enough to confer pathogenicity on it or it could be because of the changed sequence of the *ctx* gene in EW6, the toxin it produces is defective and hence non-functional. Investigation is currently under way to distinguish between these two possibilities.

### Acknowledgements

We wish to convey our heartfelt thanks to Dr. P. J. Greenaway, Director, Molecular Genetics Laboratory, PHLS Centre for Applied Microbiology, Porton Down, UK for providing us with the CT probe and also for conveying some results prior to publication. This work was supported by the Council of Scientific and Industrial Research, New Delhi.

### References



- Casn, R. A., Music, S. I., Libonati, J. P., Schwartz, A. R. and Hornick, R. B. (1974) *Infect. Immun.*, **10**, 762.
- Craig, J. P. (1971) in *Microbial toxins* (eds. S. Kadis, T. C. Montie and S. J. Ajl) (New York: Academic Press) Vol. II A, p. 189.
- Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.*, **23**, 641.
- Gennaro, M. L., Greenaway, P. J. and Broadbent, D. A. (1982) *Nucleic Acids Res.* **10**, 4883.
- Gennaro, M. L. and Greenaway, P. J. (1983) *Nucleic Acids Res.*, **11**, 3855.
- Gerdes, J. C. and Romig, W. R. (1975) *Infect. Immun.*, **11**, 445.
- Ghosh, R. K., Siddiqui, K. A. I., Mukhopadhyay, G. and Ghosh, Amit (1985) *Mol. Gen. Genet.*, **200**, 439.
- Grunstein and Hogness (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3961.
- Hanchalay, S., Sriwatana, J., Echeverria, P., Holmgren, J., Tirapat, C., Moseley, S. L. and Taylor, D. N. (1985) *J. Clin. Microbiol.*, **21**, 288.
- Kasai, G. J. and Burrows, W. (1966) *J. Infect. Dis.*, **116**, 606.
- Kaper, J. B., Moseley, S. L. and Falkow, S. (1981) *Infect. Immun.*, **32**, 661.
- Levine, M. M., Black, R. E., Clements, M. L., Cisneros, L., Saah, A., Nalin, D. R., Gill, D. M., Craig, J. P. Young, C. R. and Ristaino, P. (1982) *J. Infect. Dis.*, **145**, 296.
- Mekalanos, J. J., Collier, R. J. and Romig, W. R. (1977) *Infect. Immun.*, **16**, 789.
- Mekalanos, J. J. (1983) *Cell*, **35**, 253.
- Mekalanos, J. J., Swartz, D. J., Pearson, G. D. N., Harford, N., Groyne, F. and de Wilde, M. (1983) *Nature (London)*, **306**, 551.
- Miller, V. L. and Mekalanos, J. J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3471.
- Mitra, S., Ghosh, A. and Ghosh, R. K. (1986) *J. Gen. Microbiol.*, **132**, 2601.
- Mukherjee, S. (1978) *Methods Microbiol.*, **12**, 51.
- Rigby, P. W., Dieckmann, M., Rhodes, C. and Berg, P. (1977) *J. Mol. Biol.*, **113**, 237.
- Siddiqui, K. A. I. and Ghosh, Amit (1983) *Biochem. Biophys. Res. Commun.*, **112**, 1106.
- Southern, E. M. (1975) *J. Mol. Biol.*, **98**, 503.
- Thomas, P. S. (1983) *Methods Enzymol.*, **100**, 255.

# RNase F and 2',5'-oligoA synthetase activities in mice after poly(I).poly(C) administration

KRISHNA BAKSI\*, BORIS SKURKOVICH and  
SIMON SKURKOVICH

Laboratory of Cell Biology and Genetics, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205, USA

**Abstract.** The present study demonstrates the presence of considerable levels of 2',5'-oligoA synthetase activity in tissue extracts from mice. The interferon inducer, poly(I).poly(C), induced the synthetase activity in all the tissue extracts *in vivo*. Similarly, a significant amount of endonuclease RNase F activity is found to be present in these tissue extracts. But interferon induction does not seem to have any significant effect on RNase F activity.

**Keywords.** RNase; 2',5'-oligoA synthetase activity; poly(I).poly(C) administration.

## Introduction

2',5'-OligoA synthetase synthesizes the oligonucleotides ppp(A2'p)<sub>n</sub>A (n = 1–4, collectively known as 2–5A) from ATP (Kerr and Brown, 1978). It is demonstrated that the basal level of 2',5'-oligoA synthetase is present in a wide variety of eukaryotic cells. The level of enzyme activity, however, varies in response not only to interferon (IFN) treatment, but also to hormones and the growth state of the cells (Hovanessian *et al.*, 1977; Ball 1979; Kimchi *et al.*, 1979; Minks *et al.*, 1979; Stark *et al.*, 1979; Krishnan and Baglioni, 1980). 2–5A activates a latent 2–5A dependent endonuclease that degrades mRNA (Hovanessian *et al.*, 1979; Clemens and Williams, 1978; Williams *et al.*, 1979; Farrell *et al.*, 1978). Most of the observations made so far with 2',5'-oligoA synthetase and RNase F are in tissue culture systems (Hovanessian *et al.*, 1977, 1979; Ball, 1979; Kimchi *et al.*, 1979; Minks *et al.*, 1979; Stark *et al.*, 1979; Krishnan and Baglioni, 1980; Clemens and Williams, 1978; Williams *et al.*, 1979; Farrell *et al.*, 1978). Thus, it will be of interest to study the effect of IFN-inducer poly(I).poly(C) on these enzyme activities in a whole animal such as mouse. The present study demonstrates that a considerable amount of 2',5'-oligoA synthetase and RNase F activities were present in tissues of mice and also shows the induction of 2',5'-oligoA synthetase only after poly(I).poly(C) administration.

## Materials and methods

### Chemicals

Oligonucleotides, poly(I).poly(C), T<sub>4</sub>RNA ligase were obtained from P. L. Biochemical Laboratories; 2',5'-ADP sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden; [<sup>32</sup>P]-pCp from ICN; HPLC grade water and methanol were from Fisher Scientific.

peritoneally. At different time intervals 4 mice bled by orbital sinus puncture, were killed and the tissue dissected out and kept frozen at  $-70^{\circ}\text{C}$  until used. Mock injected animals served as controls.

### *Preparation of tissue extract*

Tissue homogenates were prepared by homogenizing the tissue in lysis buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM  $\text{Mg}(\text{OAc})_2$ , 10 mM KCl and 7 mM  $\beta$ -mercaptoethanol in a motor-driven Potten-Elvjhem homogenizer and centrifuged for 30 min at 20,000  $g$ . The supernatant was stored at  $-70^{\circ}\text{C}$  in aliquots.

### *Synthesis of ppp(Ap)<sub>3</sub>A 321 P pCp*

High specific activity ppp(Ap)<sub>3</sub>A [ $^{32}\text{P}$ ]-pCp was synthesized by incubation 200  $\mu\text{Ci}$  5'- [ $^{32}\text{P}$ ]-pCp (lyophilized), 5  $\mu\text{l}$  T<sub>4</sub> RNA ligase, 25  $\mu\text{l}$  of ligase buffer (100 mM Hepes buffer, pH 7.6, 15 mM  $\text{MgCl}_2$ , 6.6 mM dithiothreitol, 5  $\mu\text{M}$  ribosylthymine 5'-triphosphate, 20% v/v dimethylsulphoxide) and 400  $\mu\text{M}$  of ppp(Ap)<sub>3</sub>A in a total volume of 50  $\mu\text{l}$  at  $4^{\circ}\text{C}$  for 20 h. The reaction mixture was then heated at  $90^{\circ}\text{C}$  for 5 min and centrifuged for 10 min at 10,000  $g$  to remove denatured proteins. The products were further purified by high performance liquid chromatography on  $\mu$  Bondapak C<sub>18</sub> column (Water Associates) using a methanol gradient in 50 mM ammonium phosphate buffer, pH 7.0 as described by Brown *et al.* (1981).

### *RNase F activity by radiobinding assay method*

2–10  $\mu\text{l}$  of S-10 extracts containing 10 mg/ml of protein were incubated with ppp(Ap)<sub>3</sub>A [ $^{32}\text{P}$ ]-pCp (3,000 cpm,  $2 \times 10^6 \text{Ci mon}^{-1}$  approximately) at  $0^{\circ}\text{C}$  as described for the radiobinding assay by Knight *et al.* (1980).

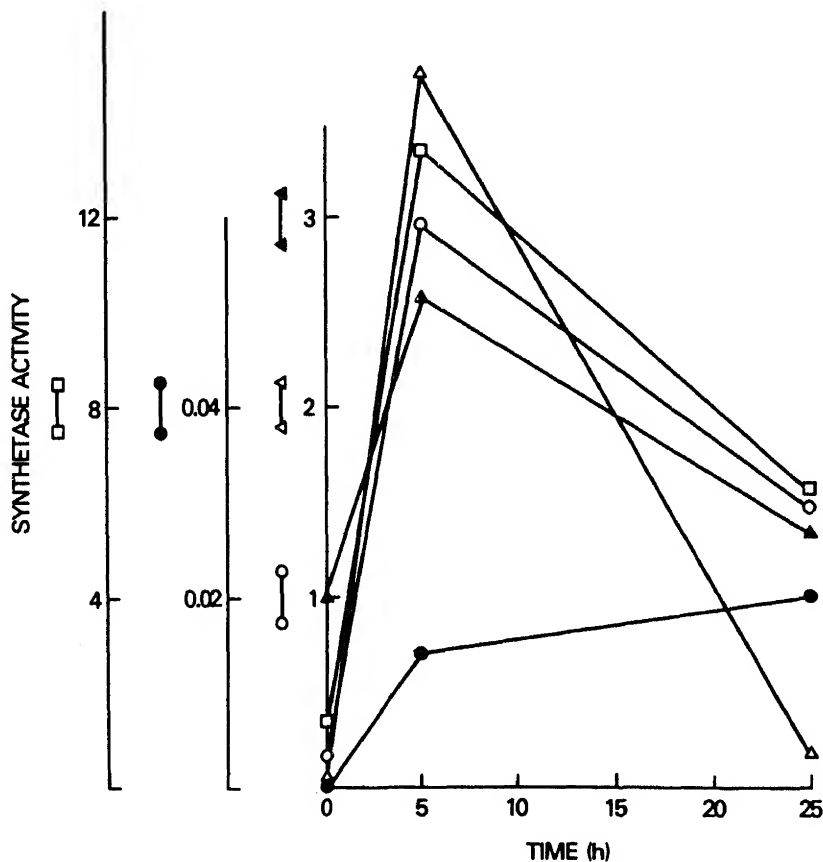
### *2',5'-OligoA synthetase assay*

Tissue extracts (25–100  $\mu\text{l}$ ) and serum (50  $\mu\text{l}$ ) were treated with 100  $\mu\text{l}$  packed 2',5'-ADP sepharose at  $4^{\circ}\text{C}$  overnight as described by Johnston *et al.* (1980). The resin-bound synthetase was incubated at  $30^{\circ}\text{C}$  in HGII-ATP buffer (20 mM Hepes, pH 7.5, 90 mM KCl, 0.5 mM  $\text{Mg}(\text{OAc})_2$ , 7 mM  $\beta$ -mercaptoethanol, 20% v/v glycerol and 5 mM Mg ATP) made  $10^{-4}\text{M}$  poly(I).poly(C) for 6 h and then centrifuged. The supernatant was heated to  $95^{\circ}\text{C}$  for 5 min and centrifuged. The heat inactivated supernatant was then assayed for its 2–5A content by radiobinding assay procedure using extracts from Ehrlich ascites tumor cells as described by Knight *et al.* (1980).

One unit of enzyme is defined as the amount of enzyme required to synthesize 1 nmol of 2–5A/h under standard assay conditions. Specific activity of the enzyme is units/mg of protein.

Protein was measured by Bio-Rad protein assay kit using crystalline bovine serum albumin as the standard.

The 2',5'-oligoA synthetase activity was measured in the tissue extracts as described in the 'materials and methods' and the results are shown in figure 1. It shows that all the tissue extracts studied contained considerable amounts of 2',5'-oligoA synthetase activity except brain where the enzyme activity could not be detected with the present assay method. Spleen showed the highest activity and the kidney the lowest. However, there was a sharp increase in the enzyme activity including brain after 5 h of intraperitoneal injection of the IFN inducer poly(I).poly(C). But there seems to be a gradual loss in the enzymatic activity in spleen, liver and lung; a rapid decrease in kidney after 25 h of poly(I).poly(C) injection. Brain still showed the elevated level



**Figure 1.** 2',5'-OligoA synthetase activity in tissue extracts after poly(I).poly(C) injection. 100  $\mu$ g poly(I).poly(C) was injected intraperitoneally and at the time indicated (hours from injection) mice were killed, tissue extracts were made and 2',5'-oligoA synthetase activity was determined as described in the 'materials and methods'. (□), Spleen; (○), liver; (▲), lung; (Δ), kidney; (●), brain.

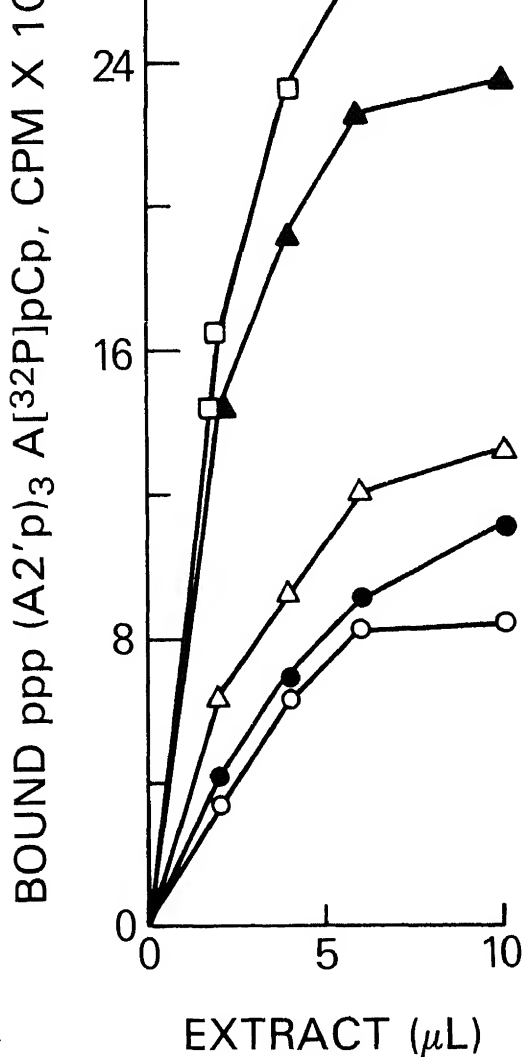
of the enzyme activity. Hence, the results indicated that IFN as produced by poly(I).poly(C) injection (Rabinovitch *et al.*, 1977) induced the 2',5'-oligoA synthetase activity similar to that has been well characterized in the IFN-treated tissue culture systems (Kerr and Brown, 1978; Hovanessian *et al.*, 1977, 1979; Ball 1979; Kimchi *et al.*, 1979; Minks *et al.*, 1979; Stark *et al.*, 1979; Krishnan and Baglioni 1980; Clemens and Williams, 1978; Williams *et al.*, 1979; Farrell *et al.*, 1978). The significance of the different level of enzyme activity in different tissues is not clear at present. However, our results are quite compatible with those of Krishnan and Baglioni (1980) with respect to the synthetase activity in spleen and lung. Although they could not detect the enzyme activity in liver and kidney, the reason for this is not clear at present. Moreover, this is the first report of detecting the 2',5'-oligoA synthetase activity in mouse brain.

2',5'-OligoA synthetase assay was also performed with the serum. But we could not detect the enzyme activity in the serum. This was not unexpected because 2',5'-oligoA synthetase activity had previously been shown only in cell extracts (Baglioni, 1979). However, there is one report on the presence of the synthetase activity in serum (Krishnan and Baglioni, 1980).

Finally, our present results together with other findings on the variations in the level of 2',5'-oligoA synthetase activity in a variety of cell and tissues (Hovanessian *et al.*, 1977; Ball, 1979; Kimchi *et al.*, 1979; Minks *et al.*, 1979; Baksi K., unpublished results), with growth and hormone status (Stark *et al.*, 1979; Krishnan and Baglioni, 1980), its ability to add 5'AMP in 2',5'-linkage to the important metabolites NAD<sup>+</sup>, ADP-ribose and A5'P<sub>4</sub>5'A (Ball and White, 1980; Ball, 1980; Ferbus *et al.*, 1981; Minks *et al.*, 1980) implies some role for the 2',5'-oligoA synthetase in regulation of cellular metabolism. Similar conclusion also reached by others (Stark *et al.*, 1979; Ball and White, 1980).

### *RNase F activity*

Tissue extracts are known to contain considerable amounts of RNA degrading enzyme(s) (Slattery *et al.*, 1979), the radiobinding assay procedure was therefore used to assay the RNase F activity in the tissue extracts. Figure 2 shows that all the tissue extracts studied here namely, liver, kidney, lung, spleen and brain contained RNaseF activity. The level of the enzyme activity again varied greatly as also was found with different cell lines (Baksi K., unpublished results). However, the highest activity was observed with spleen and the lowest with liver and brain. Not much information is available on RNase F activity in tissues mainly due to lack of a suitable assay method. Until recently, RNase F activity was assayed by measuring the RNA degradation in presence and absence of 2-5A, therefore, it could only be assayed after purification of the cells and tissue extracts containing high levels of other nucleases (Baglioni *et al.*, 1978; Patner *et al.*, 1978). Nilsen *et al.* (1981) recently have reported the radiobinding assay method of RNase L activity in tissue extracts of rabbit. Furthermore, unlike 2',5'-oligoA synthetase there was not much change in the RNase F activity in the tissue extracts after 5 h from poly(I).poly(C) injection. In all these cases, a 50–60% inhibition of binding was observed with 1 nmol of ppp(Ap)<sub>2</sub>A indicating the specificity of the enzyme activity (data not shown).



**Figure 2.** RNase F activity in tissue extracts. RNase activity was determined as described in the 'materials and methods'. (□), Spleen; (○), liver; (▲), lung; (△), kidney; (●), brain.

the absence of the synthetase activity in the serum, RNase F activity also not be detected in the serum.

In conclusion, our studies show that tissue extracts of mice contain considerable amounts of RNase F activity; the level of enzyme activity varies in different tissues. RNase F does not have much effect on this enzyme activity. Moreover, the present results together with studies by others (Nilsen *et al.*, 1981) also suggest a possible role for RNase F in the RNA metabolism.

## References

- Baglioni, C. (1979) *Cell*, **17**, 255.
- Baglioni, C., Minks, M. A. and Maroney, P. (1978) *Nature (London)*, **273**, 684.
- Ball, L. A. (1979) *Virology*, **94**, 282.
- Ball, L. A. (1980) *Ann. N. Y. Acad. Sci.*, **350**, 486.
- Ball, L. A. and White, C. N. (1980) in *Regulation of macro-molecular synthesis by low molecular synthesis by low molecular weight mediators* (eds G. Koch and D. Richter) (New York: Academic Press) p. 303.
- Brown, R. E., Cayley, P. J. and Kerr, I. M. (1981) *Methods Enzymol.*, **79**, 199.
- Clemens, M. J. and Williams, B. R. G. (1978) *Cell*, **13**, 565.
- Farrell, P. J., Sen, G. C., Dubois, M. F., Ratner, L., Slattery, E. and Lengyel, P. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 5893.
- Ferbus, D., Justesen, J., Besancon, F. and Thung, W. N. (1981) *Biochem. Biophys. Res. Commun.*, **100**, 847.
- Hovanessian, A. G., Brown, R. E. and Kerr, I. M. (1977) *Nature (London)*, **268**, 537.
- Hovanessian, A. G., Wood, J., Meurs, E. and Montagnier, L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3261.
- Johnston, M. L., Friedman, R. M. and Torrence, P. J. F. (1980) *Biochemistry*, **19**, 5580.
- Kerr, L. M. and Brown, R. E. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 256.
- Kimchi, A., Shulman, L., Schmidt, S., Chernajovsky, Y., Fradin, A. and Revel, M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3208.
- Knight, M., Cayley, P. J., Silverman, R. H., Wreschner, D. H., Gilbert, C. S., Brown, R. E. and Kerr, I. M. (1980) *Nature (London)*, **288**, 189.
- Krishnan, I. and Baglioni, C. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 6506.
- Minks, M. A., Benveniste, S., Maroney, P. A. and Baglioni, C. (1979) *J. Biol. Chem.*, **254**, 5058.
- Minks, M. A., Benveniste, S. and Baglioni, C. (1980) *J. Biol. Chem.*, **255**, 5031.
- Nilsen, T. W., Wood, D. L. and Baglioni, C. (1981) *J. Biol. Chem.*, **256**, 10751.
- Patner, L., Wiegand, R. C., Farrell, P. J., Sen, G. C., Carber, B. and Lengyel, P. (1978) *Biochem. Biophys. Res. Commun.*, **81**, 947.
- Rabinovitch, M., Manejias, R. E., Russo, M. and Abbey, E. E. (1971) *Cell Immunol.*, **29**, 86.
- Slattery, E., Ghosh, N., Samanta, H. and Lengyel, P. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4478.
- Stark, G. R., Dower, W. J., Schimke, R. T., Brown, R. E. and Kerr, I. M. (1979) *Nature (London)*, **278**, 469.
- Williams, B. R. G., Golgher, R. R. and Kerr, I. M. (1979) *FEBS Lett.*, **105**, 47.

# Histochemical localization of protein-polysaccharides in renal tissue

S. SARASWATHI\*, J. V. TESORIERO and N. S. VASAN†

\*Anatrace Maume, Ohio

Department of Anatomy, UMDNJ, New Jersey Medical School, 100 Bergen St., Newark, New Jersey 07103, USA

**Abstract.** The purpose of this study was to investigate the distribution of protein-polysaccharides in the glomerular and non-glomerular regions of the nephron. The techniques used include the digestion of kidney slices with specific polysaccharidases: neuraminidase, hyaluronidase, chondroitinase ABC, and collagenase followed by several cytochemical techniques to identify the glycosaminoglycans and glycoproteins at the light and electron microscope levels. Differential staining of hyaluronic acid and sulphated glycosaminoglycans was accomplished with Alcian Blue at pH 2.5 and pH 0.5, respectively. Sialoproteins were stained with Alcian Blue at pH 2.5. The periodic acid Schiff's reaction technique was employed for the visualization of collagen. At the electron microscope level the polysaccharides were identified with the periodic acid-chromic acid-silver methenamine reaction. Our results indicated that the major polysaccharide components of the glomerular basement membrane were sialoproteins and collagen, with smaller amounts of hyaluronic acid and various sulphated glycosaminoglycans. Hyaluronidase digestion resulted in partial detachment of epithelial processes from the glomerular basement membrane indicating the hyaluronic acid may have a role in the stability of the attachment of these processes. Tubular basement membranes also contain sialoproteins and sulphated glycosaminoglycans but in considerably lower concentrations than the glomerular basement membrane. Bowman's capsule appears to contain mostly sulphated glycosaminoglycans and has a lower concentration of sialoproteins and hyaluronic acid.

**Keywords.** Kidney; glycosaminoglycans; histochemistry.

## Introduction

Mammalian kidneys contain a spectrum of protein-polysaccharides consisting of collagen, sialoproteins, and glycosaminoglycans (GAGs) (Allalouf *et al.*, 1964; Castor and Greene, 1968; Farber and Van Praag, 1970; Murata, 1976; Linker *et al.*, 1981). The structural and functional roles of these glycoconjugates in renal tissue (Pinter, 1967; Kanwar and Farquhar, 1979) and their alterations in disease conditions such as diabetes (Berenson *et al.*, 1970; Malathy and Kurup, 1972; Saraswathi and Vasan, 1982) has been of considerable interest in recent years.

Biochemical studies using solubilized glomerular basement membrane (GBM) have provided considerable information as to the protein-polysaccharide composition of this region in normal and in pathological conditions (Mohos and Skoza, 1969; Kefalides, 1974; Spiro, 1976; Brownlee and Spiro, 1979; Parthasarathy and Spiro, 1980). Other studies using cationic probes (Kanwar and Farquhar, 1979a, b; Reeves *et al.*, 1980) as well as immunological techniques (Carlson *et al.*, 1978; Schienman *et al.*, 1978; Oberley *et al.*, 1979; Madri *et al.*, 1981) have explored the distribution of these materials in various regions of the nephron. The present study

†To whom all correspondence should be addressed.

Abbreviations used: GAGs, Glycosaminoglycans; GBM, glomerular basement membrane; PAS, periodic



further examines the distribution of several glucoconjugates in glomerular and non-glomerular regions of the nephron. The techniques employed for this investigation involve the digestion of renal tissue with specific polysaccharidases followed by several cytochemical techniques for GAGs and glycoproteins at the light and electron microscopic levels. Our results indicate that the glucoconjugates are distributed in varying concentrations in different regions of the nephron, with the GBM being rich in sialoproteins, collagen, and sulphated as well as non-sulphated GAGs.

## Materials and methods

Protease-free collagenase was purchased from Advanced Biofacture Inc., Lynbrook, New York. Chondroitinase ABC was obtained from Miles Laboratory, Indiana. Leech hyaluronidase was bought from Biomatrix Corporation, Boston, Massachusetts, and *Vibrio Cholerae* Neuraminidase (Protease free) from Calbiochem, California. Prior to use, the enzymes were tested for activity by standard procedures (Ada *et al.*, 1961; Yuki and Fishman 1963; Saito *et al.*, 1968; Gisslow and McBride, 1975). Proteolytic activity was tested using bovine serum albumin as substrate and indicated that the above enzymes were free of proteases. Hexamethylene tetramine (methenamine) was obtained from Polyscience Lab, Warrington, Pennsylvania.

Adult male Lewis rats, weighing nearly 200 g, were sacrificed under ether anesthesia. The kidneys were removed, stripped of their capsule, and cross sectioned to about 1 mm slices. The tissue slices were incubated for 14–16 h, in one of the following enzyme solutions:

- (i) *Vibrio cholerae* neuraminidase in 0.05 M sodium acetate buffer pH 5.6, containing 0.002 M  $\text{CaCl}_2$ .
- (ii) Leech hyaluronidase in McIlwains buffer, pH 5.6.
- (iii) Chondroitinase ABC (*P. Vulgaris*), in 0.1 M Tris-HCl buffer, pH 7.8.
- (iv) Collagenase in 0.025 M Tris–0.33 M calcium acetate buffer, pH 7.8.

Addition of various enzymes was repeated every 4 h. Control experiments, where the kidney slices were incubated either in physiological saline or the appropriate buffers without the enzymes, were also performed.

## Light microscopy

After the incubations, the tissue slices were rinsed in buffer, and fixed in buffered formalin for 24 h. The tissues were well rinsed in buffer, dehydrated with alcohol, and embedded in paraffin.

Sections (6  $\mu\text{m}$ ) were mounted on glass slides, and stained according to one of the following methods: Alcian Blue (8GX 300) at pH 2.5 for non-sulphated polysaccharides (Hyaluronic acid, and sialoproteins); Alcian Blue (8GX 300) at pH 0.5 to

fixed in glutaraldehyde (2%), dehydrated with ethanol, and embedded in Araldite. Semi-thin sections of about 200  $\mu\text{m}$  were picked up on nickel grids, and reacted with a modified periodic acid-chromic acid-silver methenamine technique for glycoproteins (PA-CA-AgMe) (Rambourg, 1967, 1971; Tesoriero, 1977, 1981). PA-CA oxidations were omitted in certain slices to serve as controls. The saline and saline incubated slices also served as morphological controls. Sections used for morphological comparison were stained with uranyl acetate and lead citrate.

## Results

### Electron microscopy

PAS staining of saline incubated control slices showed the classic distribution of PAS positive material within the glomerulus, Bowman's capsule, and in the tubular basement membrane (TBM) (figure 7). Staining with Alcian Blue at pH 0.5 indicates sulphated glycosaminoglycans are distributed predominantly in Bowman's capsule (BC) and in the TBM with notably smaller amounts in the glomerulus (figure 8). Alcian Blue staining at pH 2.5 reveals that the non-sulphated polysaccharides are present both in the glomerulus and in Bowman's capsule (figures 1 and 3). A clear distinction could be made between the sulphated and the non-sulphated polysaccharides in the tissue slices stained sequentially with Alcian Blue at pH 0.5 followed by Alcian Yellow at pH 2.5. The glomerulus stains a bright yellow indicating non-sulphated material, while BC stains a contrasting greenish blue, indicating a predominance of sulphated material with a smaller amount of non-sulphated polysaccharide.

Characterization of the polysaccharides, responsible for alcianophilia under different conditions, was achieved by selective digestion with specific polysaccharidases.

Tissue slices incubated with neuraminidase showed a marked reduction in the Alcian Blue (pH 2.5) staining within the glomerulus and Bowman's Capsule indicating that these regions are rich in sialoproteins (figure 2). After digestion, the GBM resembles a fine line rather than its more robust pre-digestion staining (figures 2 and 4). Digestion with leech hyaluronidase resulted in a slight to moderate reduction in Alcian Blue (pH 2.5) stain in the glomerular regions (figure 4). Since this enzyme specifically hydrolyzes hyaluronic acid, the present observation is indicative of the presence of moderate concentrations of hyaluronic acid in the glomerulus.

Following digestion with chondroitinase ABC, the intensity of the Alcian Blue (pH 0.5) stain was reduced in BC and in the TBM (figure 6). The presence of moderate amounts of chondroitin sulphate or dermatan sulphate in these regions is indicated since chondroitinase ABC specifically digests these sulphated GAGs. The persistent staining for sulphated polysaccharides, even after the digestion with chondroitinase ABC, probably indicates the presence of heparan sulphate which is not removed by chondroitinase ABC enzyme.

The digestion with collagenase often showed marked morphological changes in the tissue but only slight reduction in the PAS staining of the glomerulus.

Kidney slices which were incubated in saline for up to 24 h and processed by routine methods for electron microscopy showed only slight changes in the general architecture of the epithelial and endothelial cells of the glomerulus (figure 9). Their attachment to the GBM was maintained and the general appearance of all basement membranes appeared unchanged.

When semi-thin sections (200  $\mu\text{m}$ ) of saline incubated tissue slices were reacted according to the PA-CA-Ag methenamine technique for glycoproteins there was a positive reaction over the GBM, around the processes of the epithelial and endothelial cells, within the mesangial regions, within the endothelial cells of BC and over the basement membrane of BC (figure 10). Occasional isolated fibers of collagen which stand out strongly with this technique were noted on the mesangial side of the GBM.

Following neuraminidase digestion the silver methenamine reaction for glycoprotein was almost totally abolished over the GBM and from the surface of the endothelial cells. A slight reactivity remained around the processes of the epithelial cells. Sialoglycoproteins therefore appear to be the predominant polysaccharides in these regions (figure 11). The reactivity of BC was reduced, and the silver reactions appeared over small, fibrillar, linear structures. The heavier collagen associated with BC remained reactive. These findings suggest that moderate amounts of sialoproteins and collagen are associated with BC (figure 14).

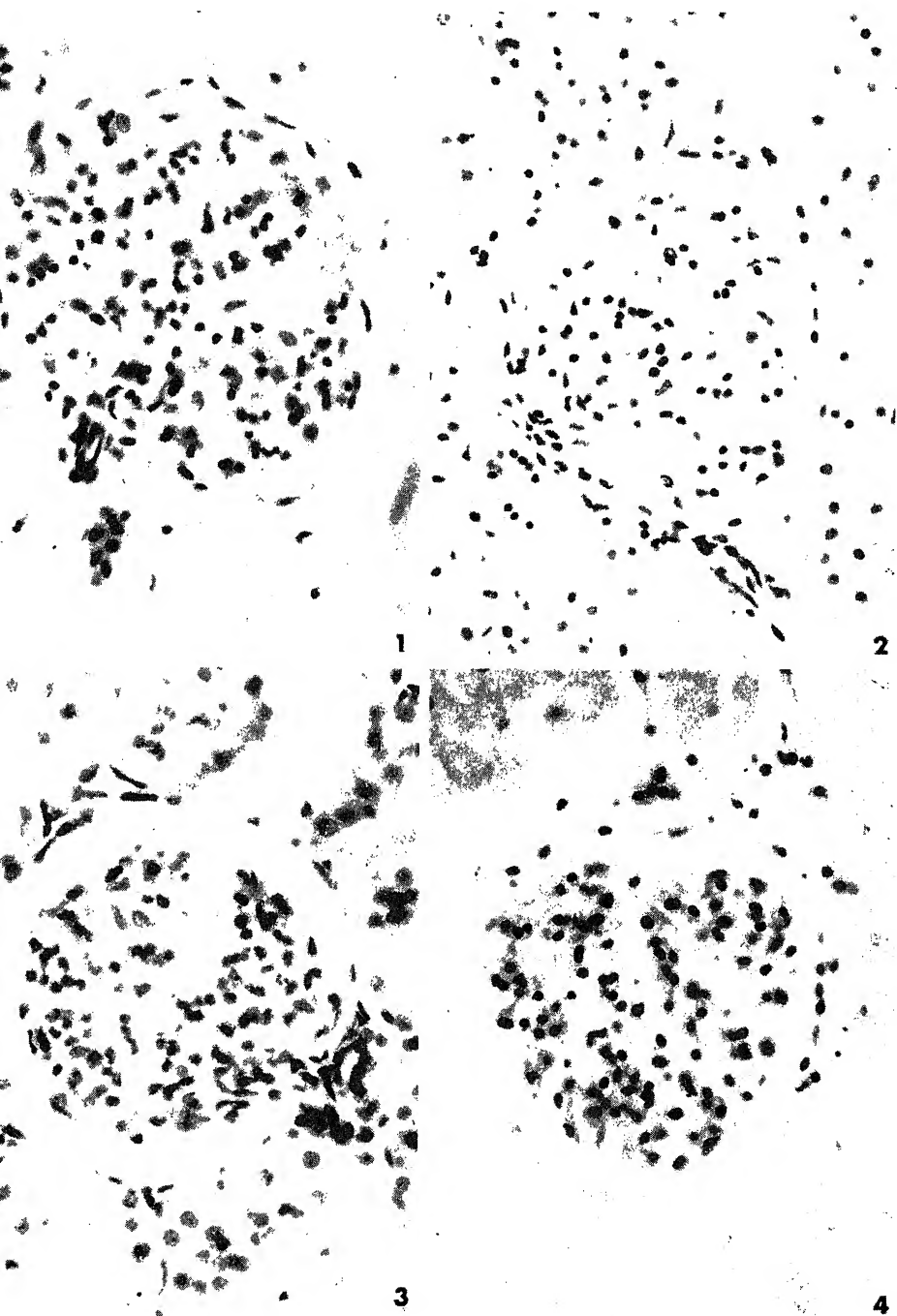
Hyaluronidase digestion resulted in a reduction in the silver methenamine reaction over most of the GBM indicating the presence of hyaluronic acid in this region. The reactivity surrounding the endothelial and epithelial cells and their processes remained unchanged. In the region of the GBM there was an electron lucent and less reactive area within regions of the lamina rara interna (LRI), which appeared to represent a separation of the epithelial cells from the membrane (figure 12). A similar region was also seen but less consistently on the endothelial side of the GBM. Hyaluronic acid may therefore have a role in the makeup of the GBM and the attachment of these cells to that membrane.

Digestion with chondroitinase ABC resulted in the reduction of silver staining in the lamina rara externa and LRI of the GBM indicating the presence of some chondroitin and/or dermatan sulphate in these regions (figure 13). BC, the epithelial cell processes, and the lamina dense portion of the GBM were still reactive to the silver stain. These regions appear to contain very little of the chondroitinase ABC digestible GAG.

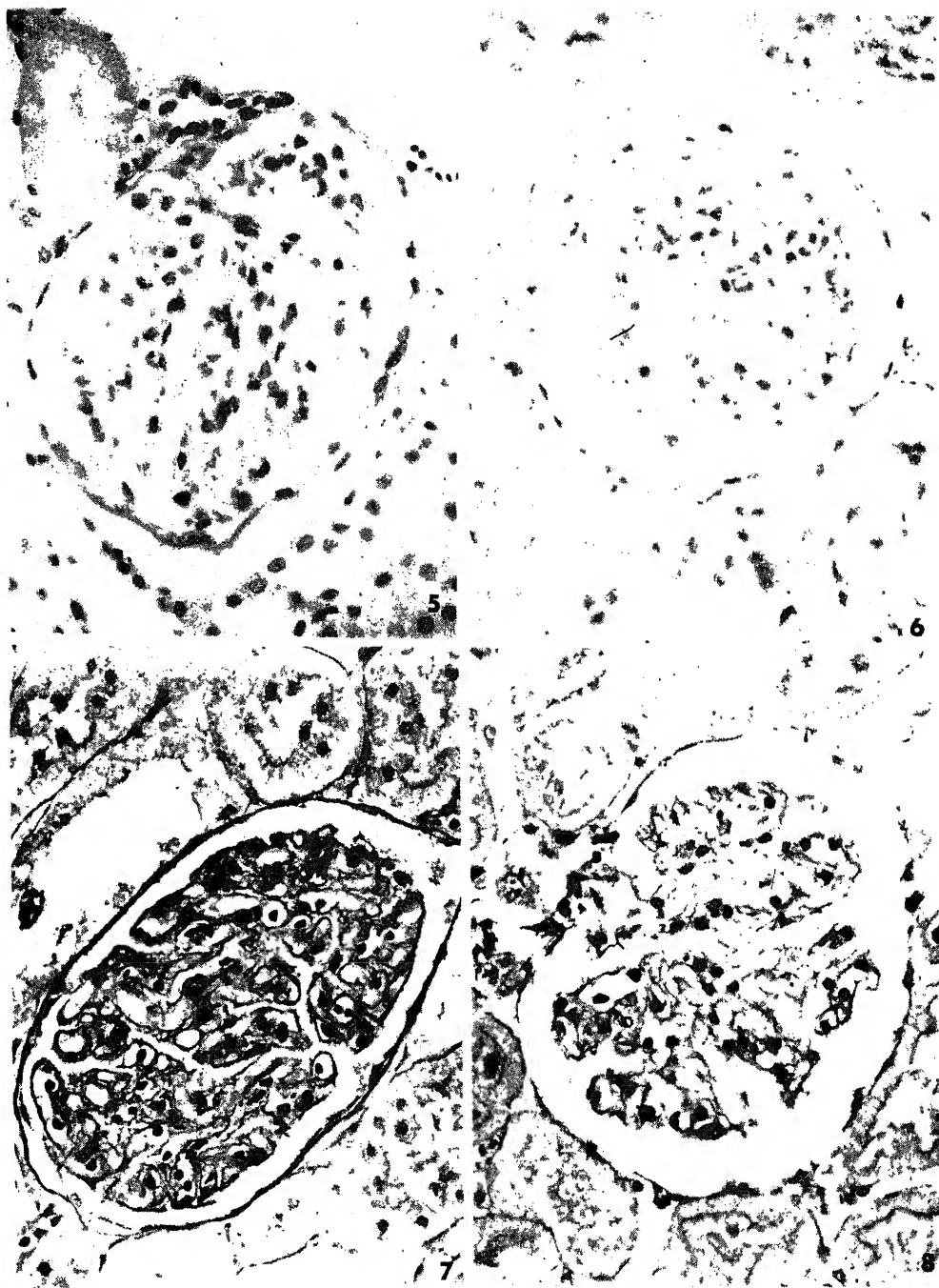
There were major structural changes in the renal tissue following digestion with collagenase but an occasional intact glomerulus could be located. The GBM retained most of its reactivity to the silver stain, as did the epithelial and endothelial cells. BC showed some loss of silver staining, with the notable absence of the highly reactive collagen.

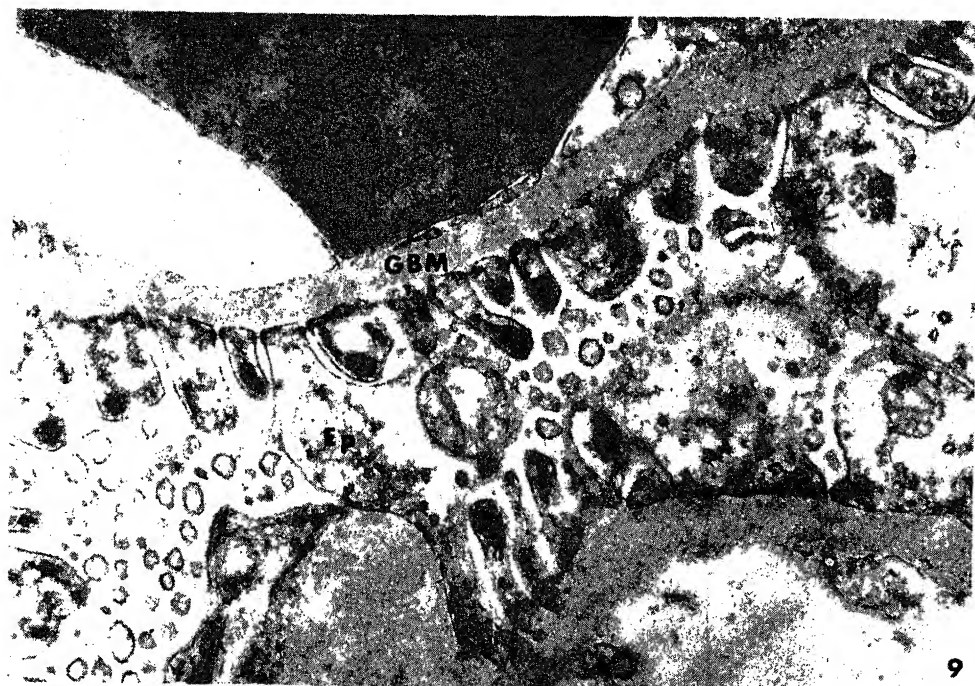
## Discussion

The enzymatic digestion, as well as light and electron microscopic studies, clearly

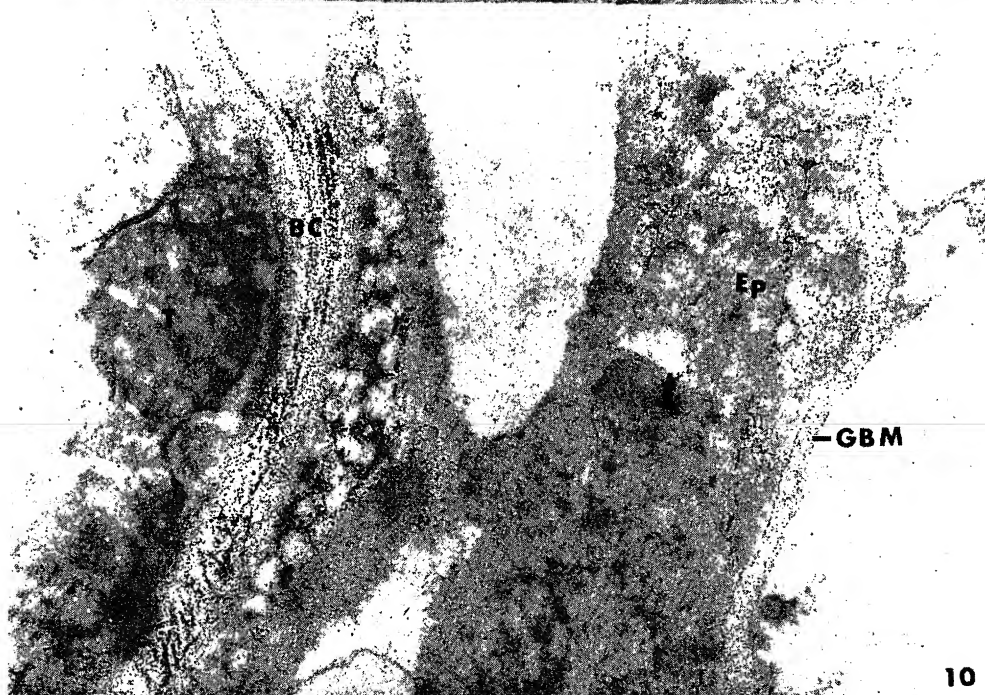


Figures 1-4. 1. Saline incubated control, Alcian Blue, pH 2.5,  $\times 135$ . 2. Neuraminidase



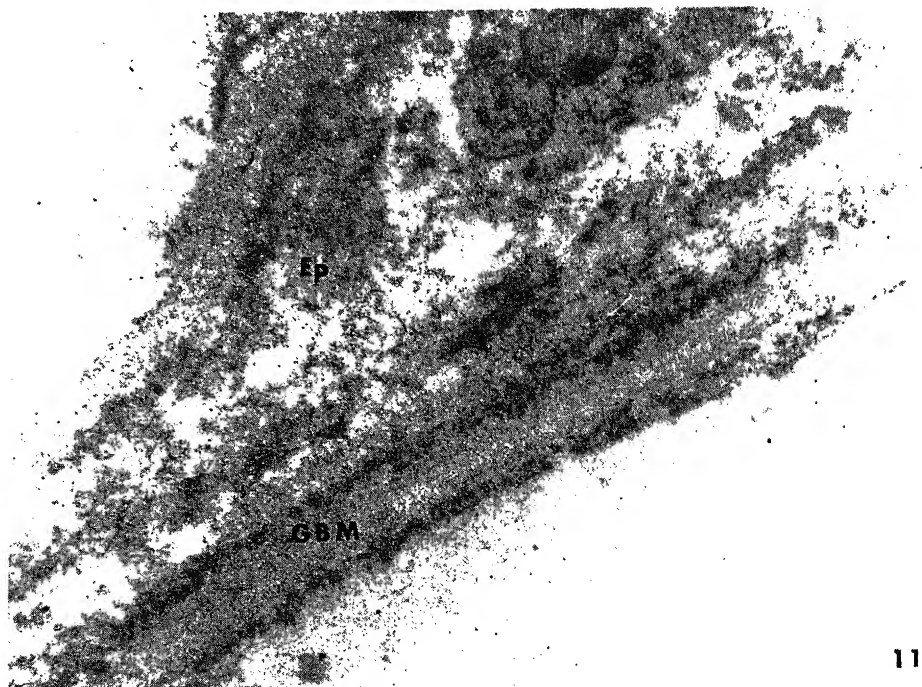


9

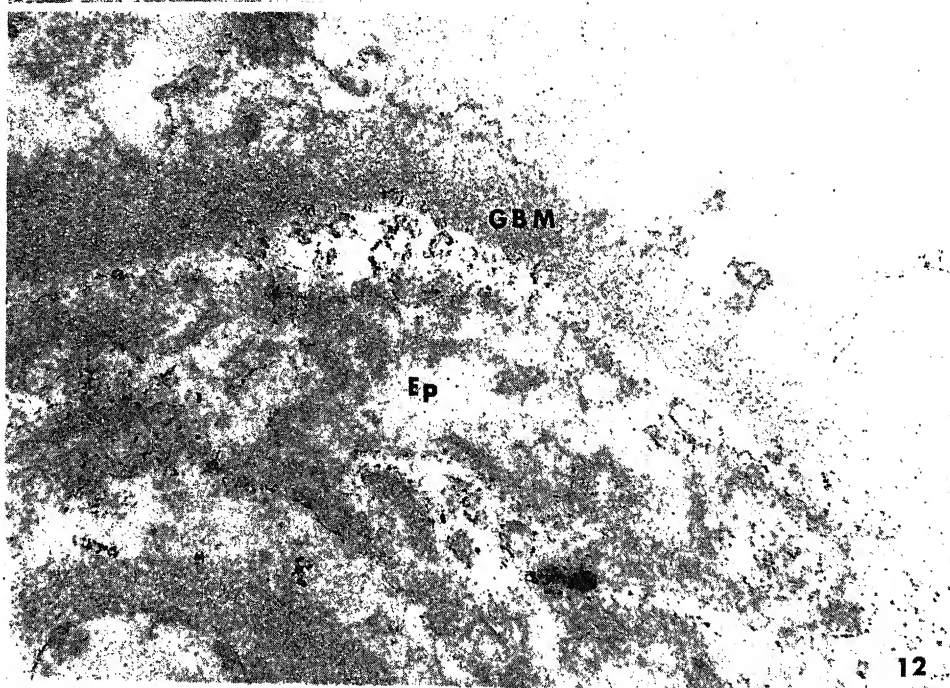


10

Figures 9 and 10. 9. Saline incubated control. Pb citrate.  $\times 25,000$ . 10. Control section



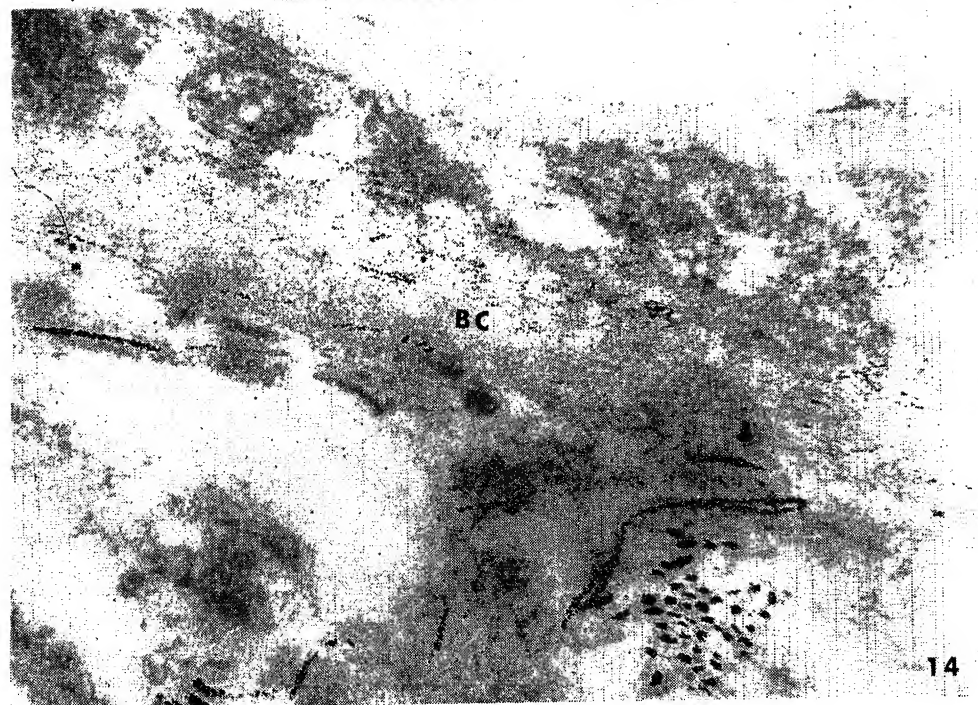
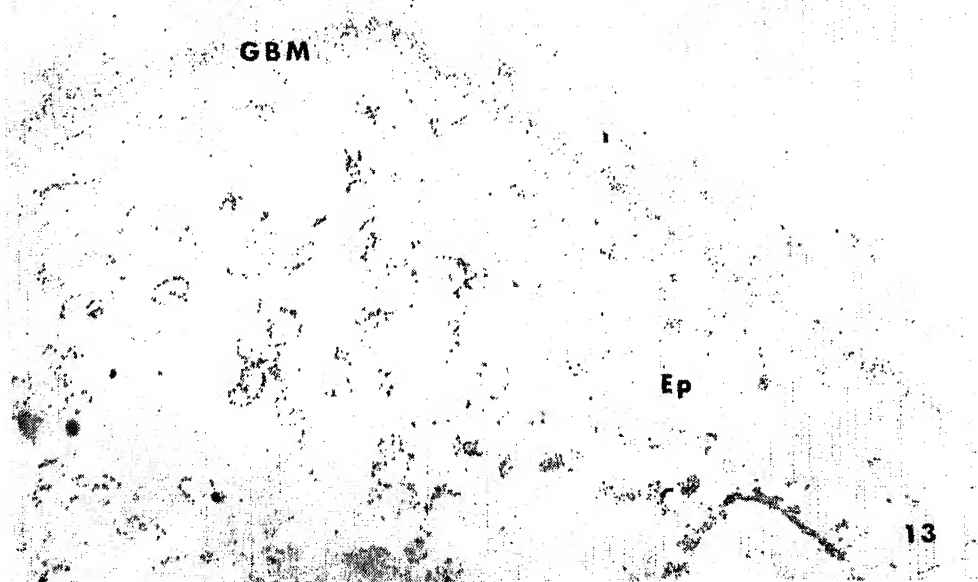
11



12

Figures 11 and 12. 11. Neurominidase digestion. PA-GA-Meth.  $\times 40,000$ . 12. H. et al.





**Figures 13 and 14.** 13. Chondroitinase ABC digestion, PA-CA-Meth.,  $\times 40,000$ . 14. Neuraminidase digestion, Bowman's capsule, PA-CA-Meth.,  $\times 40,000$ . Ep, Epithelial cells; GBM,



was found to contain considerable amounts of sialoproteins located primarily in the GBM, and on the surface of epithelial and endothelial cells. Studies using enzyme perfusion methods, as well as cationic probes have previously identified the presence of sialoproteins on epithelial and endothelial surfaces (Mohos and Skoza, 1969; Andrews, 1978; Kanwar and Farquhar, 1979a, 1980) but failed to identify the sialoproteins within the GBM. Kanwar and Farquhar (1980) have reported that the removal of sialic acid from the above regions, by means of perfusion with neuraminidase, resulted in the detachment of these processes from the GBM. We did not observe such alterations in the ultrastructure of the glomerulus resulting from the enzymatic removal of sialic acid (figure 11).

Identification of sialoproteins within the GBM has been primarily based on the analysis of solubilized GBM (Sato *et al.*, 1975; Spiro, 1976). Recently, the use of specific antibodies has (Madri *et al.*, 1981) identified the presence of sialoproteins such as fibronectin and laminin within the GBM in mesangial regions (Oberley *et al.*, 1979; Courtoy *et al.*, 1980; Madri *et al.*, 1981). The present study provides additional data which demonstrates the presence of these anionic polysaccharides in GBM by means of enzymatic digestion and histochemical staining.

In addition to sialoglycoproteins, we found the glomerulus to contain considerable amounts of GAGs. Even though the GAGs have been isolated and characterized from renal tissue by many investigators (Allalouf *et al.*, 1964; Castor and Green, 1964; Farber and Van Praag, 1970; Murata, 1976) the distribution of most of these macromolecules in the nephron, with the exception of heparan sulphate in GBM, has not been explored (Kanwar and Farquhar, 1979; Cohen 1980; Parthasarathy and Spiro, 1980; Linker *et al.*, 1981). The present results indicate that the GAGs which are digestible by chondroitinase ABC are predominantly located in the lamina interna, and lamina externa of GBM. The presence of chondroitin sulphate and dermatan sulphate in these regions, as indicated by this observation, has not hitherto been reported. The GBM still retained some reactivity to Alcian Blue (pH 0.5) stain for sulphated GAG, even after digestion with chondroitinase ABC, indicating the presence of heparan sulphate.

In addition to the sulphated GAGs, the GBM was also found to contain small amounts of hyaluronic acid, as indicated by the reduction of silver-methenamine stain, upon incubation with hyaluronidase. The presence of this GAG in GBM extracts has been suggested previously (Kanwar and Farquhar, 1979), but has not been confirmed until now. The reduction in the silver-methenamine stain was most notable at the interface of the GBM and the endothelial process. Upon the removal of hyaluronic acid, this region was often found to be unstable indicating that this GAG may have a role in the stability of the attachment of these processes to the GBM.

Heparan sulphate has been implicated as the anionic site of the GBM, regulating the filtration of anionic plasma proteins (Kanwar and Farquhar, 1979a). While the role of chondroitin sulphate and dermatan sulphate in GBM is not clearly understood, these sulphated GAGs are known to influence the fibrillogenesis of collagen (Obrink, 1973). The presence of collagen in the GBM, as noted in the electron microscopic studies as well as by the earlier reports (Westberg and Michael, 1973; Trelstad and Lawlye, 1977). suggests that this protein and the GAGs may have

Although collagen has been identified in isolated GBM and BC, it is not usually demonstrable in electron micrographs. The PA-CA-Ag methenamine technique is very reactive for collagen and we have found isolated strands of collagen fibers located on the epithelial and mesangial side of GBM, and throughout the width of BC (figure 14).

The present study also demonstrates the polysaccharide composition of TBM and BC. As has been reported earlier (Munakata *et al.*, 1978) in isolated TBM, our study indicated the presence of sialoglycoproteins in this region. The concentration of this macromolecule was found to be somewhat lower in this region than the GBM which is in accord with earlier biochemical studies (Langeveld and Veerkamp, 1981). In addition to sialoproteins, TBM also contained sulphated GAGs, which were mostly digestible by chondroitinase ABC. It is not clear whether these glucoconjugates of TBM have a role in the tubular reabsorption process during urine production.

BC has a lower content of sialoproteins and hyaluronic acid than GBM. This region appeared to contain predominantly sulphated GAG, especially HS. The significance of this finding is not immediately understood. Nevertheless, a knowledge of the distribution of various polysaccharides at the ultrastructural level will be valuable in future studies on the role of these macromolecules in normal and pathological renal tissues.

## Acknowledgements

This work was supported by the New York Diabets Association and by NIH 5-SO7RRO5393. The authors wish to thank Ms. Kimberly Alexander and Ms. Deborah Shelton, who were supported by the Rockefeller Foundation-Howard University Program, for their technical assistance.

## References

- Ada, G. L., French, E. L. and Lind, P. E. (1961) *J. Gen. Microbiol.*, **24**, 409.  
Allalouf, D., Ber, A. and Sharon, N. (1964) *Biochim. Biophys. Acta*, **83**, 278.  
Andrews, P. M. (1978) *Am. J. Anat.*, **153**, 291.  
Berenson, G. S., Ruiz, H., Dalferes, E. R., Dugan, F. A. and Radhakrishnamurthy, G. (1970) *Diabetes*, **19**, 161.  
Brownlee, M. and Spiro, R. G. (1979) *Diabetes*, **28**, 21.  
Carlson, E. C., Brendel, K., Hjelle, J. T. and Meezan, E. (1978) *J. Ultrastruct. Res.*, **62**, 26.  
Castor, C. W. and Greene, J. A. (1968) *J. Clin. Invest.*, **47**, 2125.  
Cohen, M. P. (1980) *Biochem. Biophys. Res. Commun.*, **92**, 343.  
Courtroy, P. J., Kanwar, Y. S., Hynes, R. O. and Farquhar, M. G. (1980) *J. Cell Biol.*, **87**, 691.  
Farber, S. J. and Van Praag, D. (1970) *Biochim. Biophys. Acta*, **208**, 219.  
Gisslow, M. T. and McBride, B. C. (1975) *Anal. Biochem.*, **68**, 70.  
Kanwar, Y. S. and Farquhar, M. G. (1979a) *J. Cell Biol.*, **81**, 137.  
Kanwar, Y. S. and Farquhar, M. G. (1979b) *Proc. Natl. Acad. Sci.*, **76**, 1303-1308.  
Kanwar, Y. S. and Farquhar, M. G. (1980) *Lab. Invest.*, **42**, 375.  
Kefalides, N. A. (1974) *J. Clin. Invest.*, **53**, 403.  
Langeveld, J. P. M. and Veerkamp, J. H. (1981) *Comp. Biochem. Physiol.*, **B68**, 31.  
Linker, A., Hovingh, P., Kanwar, Y. S. and Farquhar, M. G. (1981) *Lab. Invest.*, **44**, 560.  
Madri, J. A., Roll, F. J., Furthmayr, H. and Fodiat, J. M. (1981) *J. Cell Biol.*, **86**, 682.  
Malathy, K. and Kurup, P. A. (1972) *Diabetes*, **21**, 1162.  
Munakata, S. and Sato, S. (1978) *Diabetes*, **27**, 151-152.

- Murata, K. (1976) *Connect. Tiss. Res.*, **4**, 131.
- Nevins, T. E. and Michael, A. F. (1981) *Kid. Int.*, **19**, 553.
- Oberley, T. D., Mosher, D. F. and Mills, M. D. (1979) *Am. J. Pathol.*, **96**, 651.
- Obrink, E. (1973) *Eur. J. Biochem.*, **34**, 129.
- Parthasarathy, N. and Spiro, R. G. (1980) *J. Biol. Chem.*, **256**, 507.
- Pinter, G. G. (1967) *Experientia*, **23**, 100.
- Rambourg, A. (1967) *J. Histochem. Cytochem.*, **15**, 409.
- Rambourg, A. (1971) *Int. Rev. Cyt.*, **31**, 57.
- Reeves, W. H., Kanwar, Y. S. and Farquhar, M. G. (1980) *J. Cell Biol.*, **85**, 735.
- Revetto, C. (1984) *J. Histochem. Cytochem.*, **12**, 44.
- Saito, T., Yamagata, T. and Suzuki, S. (1968) *J. Biol. Chem.*, **243**, 1536.
- Sato T., Munakata, H., Yoshinaga, K. and Yoshizawa, Z. (1975) *Tohoku. J. Exp. Med.*, **115**, 229.
- Saraswathi, S. and Vasan, N. S. (1982) *Biochim. Biophys. Acta.*, **755**, 237.
- Scheinman, J. I., Fish, A. J., Matas, A. J. and Michael, A. F. (1978) *Am. J. Pathol.*, **90**, 71.
- Spiro, R. G. (1976) *Diabetologia*, **12**, 1.
- Tesoriero, J. V. (1977) *J. Histochem. Cytochem.*, **25**, 1376.
- Tesoriero, J. V. (1981) *Anat. Rec.*, **199**, 254.
- Trelstad, R. L. and Lawley, K. R. (1977) *Biochem. Biophys. Res. Commun.*, **76**, 376.
- Westberg, N. G. and Michael, A. F. (1973) *Med. Scand.*, **194**, 39.
- Yuki, H. and Fishman, W. H. (1963) *J. Biol. Chem.*, **243**, 1877.

## The positional specificities of the oxygenation of linoleic acid catalyzed by two forms of lipoxygenase isolated from Bengal gram (*Cicer arietinum*)

ALIP BORTHAKUR, B. GANESH BHAT and  
CANDADAI S. RAMADOSS\*

Biochemistry Section, Food Chemistry Department, Central Food Technological Research Institute, Mysore 570 013, India

**Abstract.** The products generated from linoleic acid by the two forms of Bengal gram lipoxygenase, BGL<sub>1</sub> and BGL<sub>2</sub>, were analysed by high-performance liquid chromatography using  $\mu$ -porasil column with isooctane containing 0.5% ethanol as the solvent system. The 13-hydroperoxyoctadecadienoic acid and its 9-isomer which are known to be produced by soybean lipoxygenase-1 and the potato enzyme respectively were used as standards. The results show that BGL<sub>1</sub> generated almost exclusively the 13-hydroperoxyoctadecadienoic acid while BGL<sub>2</sub> produced both 13- and the 9-isomer in the ratio 21:79. The secondary keto derivatives formed in the BGL<sub>2</sub> reaction were also separated by this technique.

**Keywords.** Lipoxygenase; Bengal gram.

### Introduction

Lipoxygenase (Linoleate:oxygen oxidoreductase, EC 1.13.1.13) catalyzes the oxygenation of fatty acids containing *cis*, *cis*-1,4-pentadiene system to form isomeric conjugated dienoic hydroperoxides. The enzymatic production of positional isomers, which, for linoleic acid can be either 9-hydroperoxy-10,12-octadecadienoic acid (9-LOOH) or 13-hydroperoxy-9,11-octadecadienoic acid (13-LOOH) varies with enzyme source, pH, temperature and oxygen level (Galliard, 1975). Much work has been done on the specificity of lipoxygenase from different plant species (Veldink *et al.*, 1977; Galliard and Chan, 1980) as well as of lipoxygenase isozymes from soybean (Axelrod, 1974).

Prior to the development of high performance liquid chromatographic technique (Chan and Prescott, 1975) the separation of the positional isomers was time consuming apart from its quantitative aspects being questioned (Roza and Francke, 1973). Since then, high-performance liquid chromatography (HPLC) has been extensively used for the separation of isomeric hydroperoxides (Pattee and Singleton, 1977; Chan and Levett, 1977; Yamamoto *et al.*, 1980; Shoji *et al.*, 1983; Kaplan and Ansari, 1985; Teng and Smith, 1985; Haslbeck and Grosch, 1985).

Here we report on the positional specificities for linoleate oxidation of the two forms of lipoxygenase isolated from Bengal gram.

### Materials and methods

prepared according to Bernardi (1971). A stock solution of 10 mM sodium imidazole in Tween-20 was prepared as described by Axelrod *et al.* (1981). All other reagents were of analytical grade and the solvents for HPLC were distilled and filtered before use.

### *Isolation of enzyme*

The lipoxygenase in Bengal gram extract was purified by ammonium sulphate fractionation (30–60% fraction) and DEAE-sephadex chromatography. It was resolved into two active forms by hydroxylapatite chromatography. These two forms were designated as BGL<sub>1</sub> and BGL<sub>2</sub> respectively in the order of their elution from the hydroxylapatite column. After rechromatography on hydroxylapatite both the forms appeared to be homogeneous as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The details of the purification procedure and molecular properties of BGL<sub>1</sub> and BGL<sub>2</sub> will be published elsewhere.

The enzyme was assayed either by following the oxygen consumption using a Gilson Oxygraph or by the appearance of conjugated diene hydroperoxide absorbing at 234 nm using a Beckman Model-26 Spectrophotometer. One unit of enzyme was defined as the utilisation of one  $\mu\text{mol}$  of substrate or the formation of one  $\mu\text{mol}$  of product.

Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

### *Isolation of products*

The reaction mixture (100 ml) contained 10  $\mu\text{mol}$  of linoleic acid in 0.2 M phosphate buffer, pH 6.5. The buffers were kept at 4°C and flushed with oxygen before the start of the reaction. The reaction was carried out for 15 min at 4°C with 15 units of either BGL<sub>1</sub> or BGL<sub>2</sub>. After acidifying with 2 M citric acid the products were extracted thrice with chloroform:methanol (2:1) mixture. The combined extracts were washed with water until neutral, dried over anhydrous sodium sulphate, the solvent evaporated to dryness *in vacuo* and the products dissolved in diethyl ether. The products were then methylated with diazomethane. After destroying the excess diazomethane with a few drops of 2 M acetic acid, diethyl ether was removed under a N<sub>2</sub> stream and the solvent changed to isooctane. Ultraviolet spectra of the products were taken against isooctane.

### *Isolation of 9-D- and 13-L-hydroperoxy linoleic acid*

The two isomeric hydroperoxides, 9-D- and 13-L- were prepared by aerobic incubation of linoleic acid (10  $\mu\text{mol}$ ) with potato extract at pH 6.5 (Galliard and Phillips, 1971) and soybean extract at pH 9.0 (Garssen *et al.*, 1971) respectively. The conditions for the enzymatic reactions, isolation of the products and their methylation were similar to those described for Bengal gram lipoxygenase products.

Methylated lipoxygenase products were analysed by HPLC using a Waters Associates Liquid Chromatograph (Milford, Massachusetts, USA) equipped with a 6000 A pump, U6K injector and Model 441 absorbance UV-detector. The conditions for HPLC were: column  $\mu$ Porasil (30 cm  $\times$  3.9 mm I.D.); mobile phase, 0.5% anhydrous ethanol in isooctane; flow rate, 2 ml/min; detection, 229 nm for hydroperoxides and 280 nm for ketodienes.

### Mass spectrometry

The HPLC purified products after removal of solvent under *vacuo* were reduced with  $\text{NaBH}_4$  in methanol. The reaction mixture was acidified with 2 M acetic acid, extracted with chloroform, and the chloroform evaporated under a stream of  $\text{N}_2$ . The dried products were kept at  $-20^\circ\text{C}$  until used for analysis. Mass spectra of the products were recorded with a Hewlett-Packard 5995B GC-MS instrument using the direct insertion probe.

### Thin-layer chromatography

The HPLC purified lipoxygenase products were reduced with  $\text{NaBH}_4$  and then analysed by thin-layer chromatography (TLC) on silica gel G plates using the solvent system petroleum ether (60–80°C):diethyl ether:acetic acid (80:20:1, v/v/v). The spots were visualized by spraying with 5% (w/v) phosphomolybdic acid in 95% (v/v) ethanol followed by heating at  $110^\circ\text{C}$  (Garssen *et al.*, 1971).

## Results and discussion

The lipoxygenase activity present in Bengal gram was resolved into two forms. The two forms, designated as  $\text{BGL}_1$  and  $\text{BGL}_2$  were shown to be kinetically distinct (Borthakur and Ramadoss, 1986).  $\text{BGL}_1$  catalyzed the oxidation of linoleic acid exclusively to the diene hydroperoxide which absorbs maximally at 234 nm. The  $\text{BGL}_2$  reaction produced ketodienes also in addition to hydroperoxide derivative and the product showed absorption maxima at 234 and 278 nm.

In order to determine the regiospecificities of the reactions of  $\text{BGL}_1$  and  $\text{BGL}_2$ , the products were analysed by HPLC. It is known that at pH 9.0 the soybean enzyme generates essentially the 13-hydroperoxyoctadecadienoic acid (Garssen *et al.*, 1971) while the potato enzyme generates predominantly the 9-isomer (Galliard and Phillips, 1971). These two compounds were prepared using crude enzymes from soybean and potato for use as standards. The products from the two enzymes were methylated and chromatographed separately and as 1:1 mixtures. As shown in figure 1A there is an excellent separation of the two isomers with retention times of 11 and 13.6 min respectively for the 13- and 9-isomers.

The solvent system used appeared to be quite comparable to the hexane:ethanol

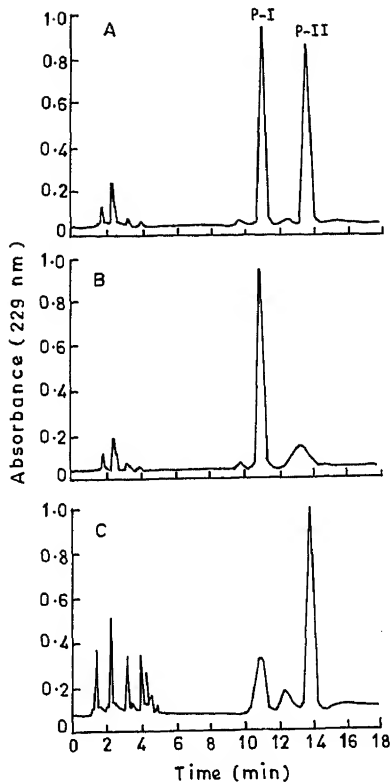


Figure 1. HPLC of methylated linoleate hydroperoxides formed by soybean and potato lipoxigenase (A), BGL<sub>1</sub> (B) and BGL<sub>2</sub> (C) on  $\mu$ Porasil column (300  $\times$  3.9 mm); mobile phase, 0.5% ethanol in isooctane; flow rate, 2 ml/min and UV detection at 229 nm. Component identities: peak-I, 13-LOOH; peak-II, 9-LOOH.

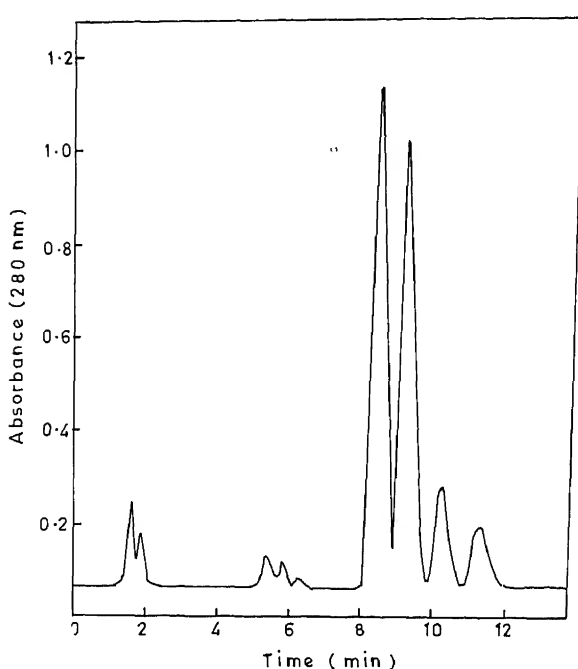
must be anhydrous as the presence of moisture markedly affects reproducibility of the chromatograms.

The proportion of the 13- and 9-isomers were 91:9 and 5:95 respectively for the soybean and potato enzymes and in agreement with earlier reports (Galliard, 1975).

The HPLC analysis of the BGL<sub>1</sub> and BGL<sub>2</sub> products are also shown in figure 1. The BGL<sub>1</sub> reaction product had the same retention time as that of the soybean lipoxigenase product (figure 1B). The minor peak corresponded to the potato enzyme product. Thus, BGL<sub>1</sub> generated almost exclusively the 13-hydroperoxy derivative from linoleic acid. In this respect it appears similar to the enzyme from *Dimorphotheca sinuata* which produces only the 13-isomer at pH 6.9 (Gardner *et al.*, 1973). The soybean lipoxigenase-1 generates the 13-isomer exclusively only at pH 9.0; however, at low pH both the isomers are formed (Roza and Francke, 1973).

The HPLC analysis of BGL<sub>2</sub> product (figure 1C) showed the presence of both the 13- and the 9-isomer in the ratio of 21:79. Most of the enzymes having pH optimum below neutrality generate preferentially the 9-isomer (Galliard, 1975; Yamamoto *et al.*, 1980).

The hydroperoxide generated in the BGL<sub>1</sub> reaction was eluted in this chromatogram



**Figure 2.** HPLC of methylated ketodienes of linoleic acid formed by BGL<sub>2</sub>. Mobile phase, 0.2% ethanol in isooctane and other conditions as in figure 1.

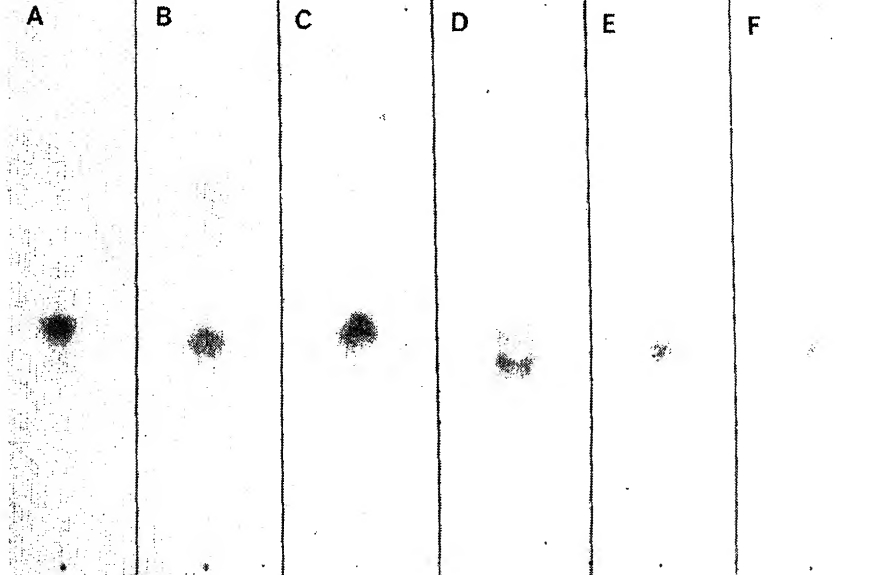
tration in the mobile phase was reduced to 0.2% the keto derivatives separated into two components (figure 2). In TLC also the ketodiene fraction was found to be a mixture of two closely migrating components.

The UV spectra of the BGL<sub>1</sub> products before and after HPLC separation showed absorption only at 234 nm. The BGL<sub>2</sub> products on the other hand showed two peaks at 234 and 278 nm before HPLC separation. After HPLC separation, the hydroperoxide fraction showed only the 234 nm absorption peak. While the keto derivatives showed absorption only at 278 nm. On NaBH<sub>4</sub> reduction of ketodienes the 278 nm peak disappeared but a new peak appeared at 234 nm for the hydroxy diene chromophore.

TLC comparison of the NaBH<sub>4</sub> reduction products of the two ketodienes separated by HPLC with the NaBH<sub>4</sub> reduction products derived from soybean, potato and BGL<sub>1</sub> enzyme products indicated that the first major peak (figure 2) is for 13-oxooctadecadienoic acid (figure 3). The second major ketodiene was not obtained completely pure and its reduction product had two spots corresponding to the 13- and the 9-hydroxy derivatives (figure 3). However, it is clear that the two ketodienes isolated from the BGL<sub>2</sub> catalyzed reaction were the 13- and the 9-oxo derivatives. It is likely that they arise from their respective hydroperoxy derivatives, which are the primary products.

The mass spectrum recorded for the HPLC purified soybean enzyme product





**Figure 3.** TLC separation of the HPLC purified lipoxygenase products after their  $\text{NaBH}_4$  reduction. (A), Soybean enzyme product; (B),  $\text{BGL}_1$  product; (C),  $\text{BGL}_2$  generated ketodiene-I; (D),  $\text{BGL}_2$  generated ketodiene-II, (E), potato enzyme product; (F),  $\text{BGL}_2$  generated hydroperoxides.

(M-31, loss of  $\text{OCH}_3$ ); 167 (M-143,  $(\text{CH}_2)_6\text{COOCH}_3$ ); 153 (M-157,  $(\text{CH}_2)_7\text{COOCH}_3$ ) and 125 (M-185,  $\text{CHO}(\text{CH}_2)_7\text{COOCH}_3$ ).

The results confirm the presence of 13- and the 9-isomers of hydroperoxyoctadecadienoic acid in the soybean- $\text{L}_1$  and the potato enzyme catalyzed reactions respectively.

The mass spectra of the  $\text{BGL}_1$  product (figure 1B, peak I) and the  $\text{BGL}_2$  product (figure 1C, peak II) were identical to those of the soybean- $\text{L}_1$  and the potato enzyme products respectively.

## Acknowledgements

The authors wish to thank Dr. R. Seshadri for suggesting the solvent system used here and Dr. K. N. Gurudutt for mass spectral analysis. AB and BGB thank Council of Scientific and Industrial Research, New Delhi, for the award of Fellowships.

## References

- Axelrod, B. (1974) *Adv. Chem. Ser.*, **136**, 324.
- Axelrod, B., Thomas, M. C. and Laakso, S. (1981) *Methods Enzymol.*, **71**, 441.
- Bernardi, G. (1971) *Methods Enzymol.*, **22**, 325.
- Borthakur, A. and Ramadoss, C. S. (1986) *J. Agric. Food Chem.*, **34**, 1016.
- Chan, H. W. S. and Prescott, F. A. A. (1975) *Biochim. Biophys. Acta.*, **380**, 141.

- Galliard, T. (1973) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (eds T. Galliard and E. I. Mercer) (New York: Academic Press) p. 319.
- Galliard, T. and Chan, H. W. S. (1980) in *Biochemistry of Plants—A comprehensive Treatise* (ed. P. K. Stumpf) (New York: Academic Press) vol. 4, p. 131.
- Galliard, T. and Phillips, D. R. (1971) *Biochem. J.*, **124**, 438.
- Gardner, H. W., Christianson, D. D. and Kleiman, R. (1973) *Lipids*, **8**, 271.
- Garssen, G. J., Vliegenthart, J. F. G. and Boldingh, J. (1971) *Biochem. J.*, **122**, 327.
- Haslbeck, F. and Grosch, W. (1985) *J. Food Biochem.*, **9**, 1.
- Kaplan, E. and Ansari, K. (1985) *J. Chromatogr.*, **350**, 435.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Pattee, H. A. and Singleton, J. A. (1977) *J. Am. Oil. Chem. Soc.*, **54**, 183.
- Roza, M. and Francke, A. (1973) *Biochim. Biophys. Acta*, **316**, 76.
- Shoji, I., Yuji, M. and Yuhei, M. (1983) *Agric. Biol. Chem.*, **47**, 637.
- Teng, J. I. and Smith, L. L. (1985) *J. Chromatogr.*, **350**, 445.
- Veldink, G. A., Vliegenthart, J. F. G. and Boldingh, J. (1977) *Prog. Chem. Fats Other Lipids*, **15**, 131.
- Yamamoto, A., Fujii, Y., Yasumoto, K. and Mitsuda, H. (1980) *Lipids*, **15**, 1.



# Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase

K. VARALAKSHMI, H. S. SAVITHRI and N. APPAJI RAO\*

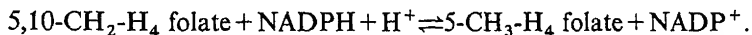
Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

**Abstract.** Cibacron Blue F3G-A, a probe used to monitor nucleotide binding domains in enzymes, inhibited sheep liver 5,10-methylenetetrahydrofolate reductase competitively with respect to 5-methyltetrahydrofolate and NADPH. The  $K_i$  values obtained by kinetic methods and the  $K_d$  value for the binding of the dye to the enzyme estimated by protein fluorescence quenching were in the range 0.9–1.2  $\mu$ M. Another triazine dye, Procion Red HE-3B interacted with the enzyme in an essentially similar manner to that observed with Cibacron Blue F3G-A. These results as well as the interaction of the dye with the enzyme monitored by difference spectroscopy and intrinsic protein fluorescence quenching methods indicated that the dye was probably interacting at the active site of the enzyme by binding at a hydrophobic region.

**Keywords.** Cibacron Blue F3G-A; Procion Red HE-3B; 5,10-methylenetetrahydrofolate reductase.

## Introduction

5,10-Methylenetetrahydrofolate (5,10-CH<sub>2</sub>-H<sub>4</sub> folate) reductase (EC 1.1.99.15), a flavoprotein catalyzes the reaction



The enzyme was earlier purified to homogeneity from sheep liver (Varalakshmi *et al.*, 1983) using affinity chromatography on Blue Sepharose matrix, a procedure similar to that described for the isolation of the enzyme from pig liver (Daubner and Matthews, 1982). The kinetic mechanism of the reaction catalyzed by the enzyme was established to be *bi-bi* ping-pong using initial velocity and product inhibition studies (Varalakshmi *et al.*, 1983) and this was in agreement with the mechanism suggested for the pig liver enzyme (Matthews and Haywood, 1979). The dye, Cibacron Blue F3G-A used as a ligand in the affinity matrix has been extensively employed to probe into the nucleotide binding domains of dehydrogenases and kinases (Rossmann *et al.*, 1974; Thompson *et al.*, 1975; Thompson and Stellwagen, 1976; Apps and Gleed, 1976; Stellwagen, 1977; Ashton and Polya, 1978; Lepo *et al.*, 1979). In addition, recent results seem to suggest that the dye could be interacting at additional sites on the protein especially in hydrophobic pockets (Chambers and Dunlap, 1979; Subramanian and Kaufman, 1980; Ramesh and Appaji Rao, 1980). The structural similarity of the dye with folate coenzymes as well as with antifolates used to probe the active site environment of dihydrofolate reductase (EC 1.5.1.3) and serine hydroxymethyltransferase (EC 2.1.2.1) suggested that the dye might interact in the region of the active site of enzymes requiring folate coenzymes as substrates. In

\*To whom all correspondence should be addressed.

Abbreviations used: 5,10-CH<sub>2</sub>-H<sub>4</sub> folate, 5,10-Methylenetetrahydrofolate; 5-CH<sub>3</sub>-H<sub>4</sub> folate, 5-methyl-

enzyme from chicken liver (Chambers and Dunlap, 1979) or in a manner which overlaps the pyridine nucleotide and dihydrofolate binding domains in the enzyme from *Lactobacillus casei* (Subramanian and Kaufman, 1980). Since 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase also binds to NADPH and 5-CH<sub>3</sub>-H<sub>4</sub> folate (Matthews and Haywood, 1979; Daubner and Matthews, 1982; Varalakshmi *et al.*, 1983), it could be predicted that the dye might interact with one of the substrate binding sites or in a manner overlapping both the sites. Procion Red HE-3B, another triazine dye has been used as an affinity ligand to purify NADP-dependent dehydrogenases (Ashton and Polya, 1978). This paper reports the interaction of sheep liver 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase with Cibacron Blue F3G-A and Procion Red HE-3B monitored by kinetic, spectroscopic and fluorimetric methods.

## Materials and methods

All the chemicals used were of analytical grade and purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Cibacron Blue F3G-A was a gift from CIBA-GEIGY (Basel, Switzerland).

### Purification

5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase was purified from sheep liver to homogeneity by ammonium sulphate fractionation, acid precipitation (pH 4.5), diethylaminoethyl-Sephacel ion-exchange and Blue Sepharose affinity chromatography (Varalakshmi *et al.*, 1983).

### Enzyme assays

Activity of 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase was measured either using radioactive [<sup>14</sup>C]-5-CH<sub>3</sub>-H<sub>4</sub> folate or by measuring the oxidation of NADPH spectrophotometrically in the presence of a suitable electron acceptor such as dichlorophenolindophenol or menadione (Varalakshmi *et al.*, 1983).

### Difference spectrum

The enzyme (1 mg/ml) was taken in sample cuvette in 0.05 M potassium phosphate buffer (pH 7.2) containing 0.3 mM EDTA and the same volume of buffer was taken in reference cuvette and the base line was recorded using a Shimadzu spectrophotometer. From a stock solution (1 mM) of Cibacron Blue or Procion Red, equal amounts of dye were added to both the cuvettes and the difference spectrum recorded. Appropriate correction for dilutions were made. When the effect of ligands on dye difference spectra was studied, the ligand (at equal concentration) was added to both the cuvettes.

Fluorescence spectra were recorded using a Perkin-Elmer model 203 fluorescence spectrophotometer, at room temperature ( $26 \pm 2^\circ\text{C}$ ) in 3 ml cuvettes. Stern-Volmer plot (Lehrer, 1971) was used to calculate the  $K_d$  values for Cibacron Blue and Procion Red dyes in fluorescence quenching experiments. The concentration of Cibacron Blue F3G-A was determined using  $\epsilon = 13.6 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ . Care was taken to ensure that the absorbance of the reaction mixture was less than 0.1 at both the excitation and emission wavelengths to eliminate internal filter effects and non-specific quenching.

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

## Results

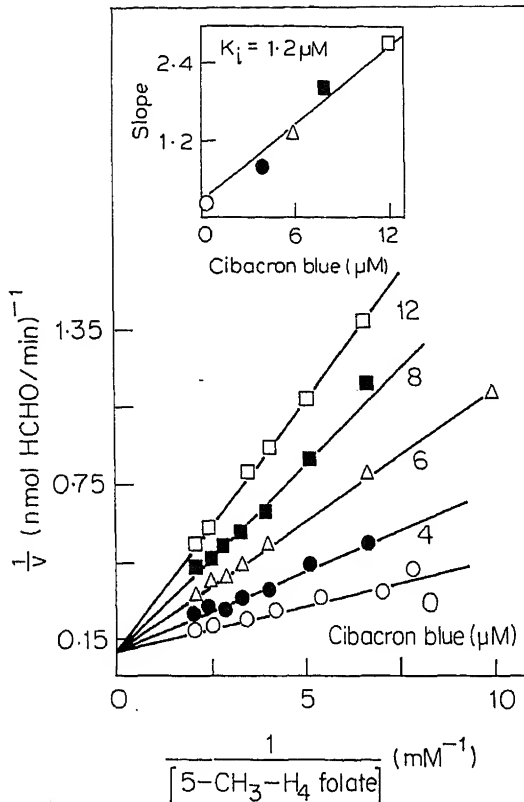
### *Kinetic analysis*

*Inhibition of the reductase by Cibacron Blue:* The inhibition of the activity of the enzyme by the dye was examined by assaying the residual activity at different fixed concentrations of Cibacron Blue (0, 4, 6, 8 and 12  $\mu\text{M}$ ) at varying concentrations of 5- $\text{CH}_3\text{-H}_4$  folate (0–10 mM). The Lineweaver-Burk plots shown in figure 1 converged at a single point on the Y-axis and a replot of the slopes of the Lineweaver-Burk plots *vs* the concentration of Cibacron Blue gave a straight line indicating that the dye was a linear competitive inhibitor with a  $K_i$  value of 1.2  $\mu\text{M}$  (inset figure 1). Similar kinetic analysis in the NADPH-menadione reductase assay (Varalakshmi *et al.*, 1983) using 0, 1, 2, 3 and 4  $\mu\text{M}$  Cibacron Blue and varying concentrations of NADPH (20–150  $\mu\text{M}$ ) showed that the dye was a linear competitive inhibitor with respect to NADPH also. A  $K_i$  value of 0.9  $\mu\text{M}$  was calculated from the slope replot (figure 2).

*Inhibition by Procion Red:* In order to compare the kinetics of the inhibition of the enzyme activity by Procion Red and Cibacron Blue, similar experiments were carried out using Procion Red. When 5- $\text{CH}_3\text{-H}_4$  folate was varied at different fixed concentrations of Procion Red (2, 4 and 6  $\mu\text{M}$ ), it showed competitive type of inhibition (figure 3). From the replot of slopes (inset figure 3), the  $K_i$  value for the dye was calculated to be 1  $\mu\text{M}$ .

### *Difference spectral studies*

Cibacron Blue F3G-A has a characteristic visible spectrum (---) with a maximum at 610 nm (figure 4A) and the enzyme has little or no absorbance between 500–800 nm. It can be seen from figure 4A, that the difference spectrum (—) has a characteristic maximum at 690 nm and a minimum around 600 nm. This characteristic difference spectrum permitted a titration of the dye binding to the enzyme. It can be seen from



**Figure 1.** Competitive inhibition of the activity of 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase by Cibacron Blue F3G-A when 5-CH<sub>3</sub>-H<sub>4</sub> folate was the varied substrate.

The enzyme (5 μg) in 0.2 M potassium phosphate buffer pH 7.2 containing 0.3 mM EDTA 0.2 M ascorbate, 50 μM FAD was preincubated with different fixed concentrations of the Cibacron Blue (○, none; ●, 4 μM; △, 6 μM; ■, 8 μM; □, 12 μM) at 37°C for 5 min and the reaction was started by the addition of various concentrations of [<sup>14</sup>C]-5-CH<sub>3</sub>-H<sub>4</sub> folate and the amount of [<sup>14</sup>C]-HCHO formed was estimated (Varalakshmi *et al.*, 1983). Inset shows a replot of the slope of the reciprocal plots *vs* the corresponding concentration of Cibacron Blue F3G-A.

Cibacron Blue (figure 4) and the  $K_d$  value (14 μM) was calculated from a double reciprocal plot (figure 4B).

The kinetic studies (figure 1) revealed that 5-CH<sub>3</sub>-H<sub>4</sub> folate was interacting competitively with Cibacron Blue. It was therefore of interest to examine whether the spectral changes induced by the dye (20 μM) upon its binding to the enzyme could be reversed by 5-CH<sub>3</sub>-H<sub>4</sub> folate. 5-CH<sub>3</sub>-H<sub>4</sub> folate (0.6, 1.0 and 1.4 mM) was added to both the sample and reference cuvettes and the spectra were recorded. 5-CH<sub>3</sub>-H<sub>4</sub> folate could reverse the difference spectrum caused by the binding of the dye to the enzyme (data not given). The concentrations of 5-CH<sub>3</sub>-H<sub>4</sub> folate were approximately in the range used for the enzyme assays (0.5 mM). At this concentration of the dye (20 μM) and 5-CH<sub>3</sub>-H<sub>4</sub> folate (0.5 mM) the enzyme activity was inhibited to more than 50% indicating that much higher concentrations may be necessary for complete

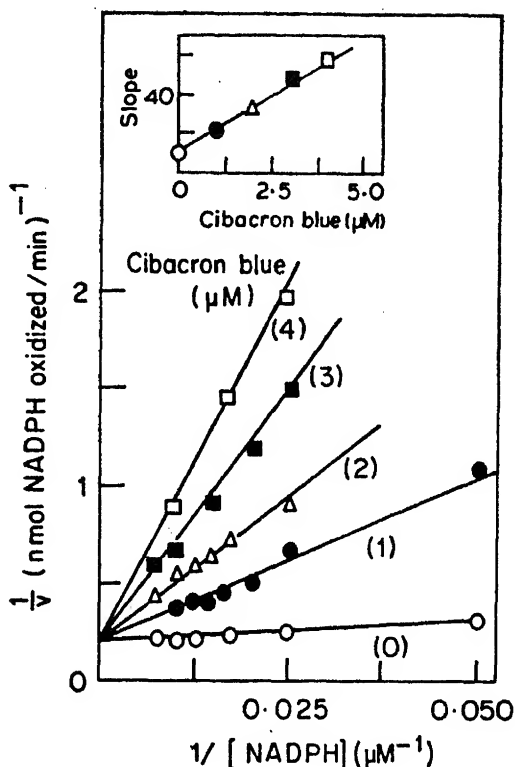


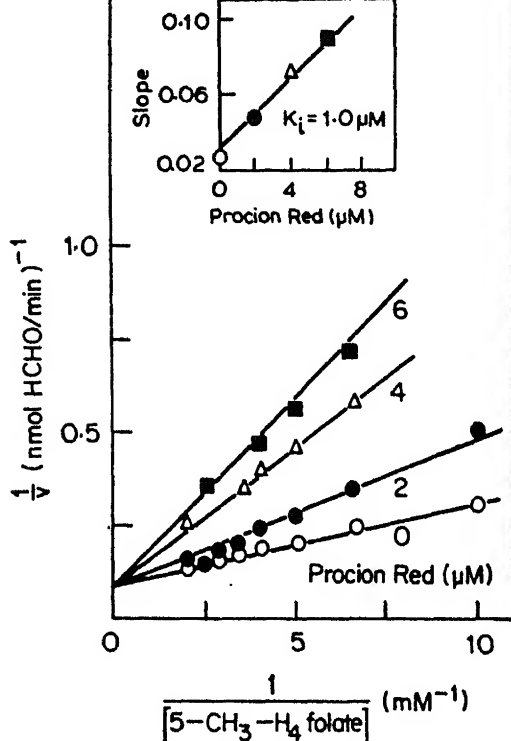
Figure 2. Inhibition of the enzyme by Cibacron Blue F3G-A when NADPH concentrations were varied.

The enzyme ( $2 \mu\text{g}$ ) in  $0.3 \text{ M}$  potassium phosphate buffer pH 7.2 containing  $0.3 \text{ mM}$  EDTA,  $0.2 \text{ mM}$  ascorbate,  $50 \mu\text{M}$  FAD was preincubated with different fixed concentrations of Cibacron Blue ( $\circ$ , none;  $\bullet$ ,  $1 \mu\text{M}$ ;  $\Delta$ ,  $2 \mu\text{M}$ ;  $\blacksquare$ ,  $3 \mu\text{M}$ ;  $\square$ ,  $4 \mu\text{M}$ ) and with various concentrations of NADPH ( $10$ – $150 \mu\text{M}$ ) for  $5 \text{ min}$  at  $25^\circ\text{C}$ . The reaction was started by adding a saturating concentration ( $125 \mu\text{M}$ ) of menadione. The absorbance change was recorded at  $343 \text{ nm}$  using a Cary 219 Recording Spectrophotometer. The amount of NADPH consumed was calculated using an  $\epsilon$  value of  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ . Inset shows the replot of slope vs Cibacron Blue concentration.

spectral changes. The elution of the enzyme from the Blue Sepharose column by  $2 \text{ M}$  KCl suggested that electrostatic interactions may also be involved in dye binding. The addition of different concentrations ( $0.5$ – $2 \text{ M}$ ) of KCl progressively decreased the intensity of the peak ( $690 \text{ nm}$ ) in the dye difference spectrum and at a concentration of  $2 \text{ M}$ , the signal disappeared completely (figure 4C).

The absorption spectrum of Procion Red exhibited two maxima at  $510 \text{ nm}$  and  $540 \text{ nm}$  whereas the difference spectrum of the dye in the presence of the enzyme showed a red shift with two peaks at  $520$  and  $570 \text{ nm}$  (data not given). This difference





**Figure 3.** Procion Red, a competitive inhibitor for the activity of 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase.

The enzyme (6  $\mu$ g) in 0.2 M potassium phosphate buffer pH 7.2 containing 0.3 mM EDTA, 0.2 M ascorbate, 50  $\mu$ M FAD was preincubated with different fixed concentrations of Procion Red dye ( $\circ$ , none;  $\bullet$ , 2  $\mu$ M;  $\triangle$ , 4  $\mu$ M;  $\blacksquare$ , 6  $\mu$ M) for 5 min at 37°C and the reaction was started by the addition of varying concentrations (0.05–0.5 mM) of [<sup>14</sup>C]-5-CH<sub>3</sub>-H<sub>4</sub> folate and the [<sup>14</sup>C]-HCHO formed was estimated (Varalakshmi *et al.*, 1983). Inset shows the replot of slopes vs Procion Red concentration.

ligands is the quenching of intrinsic protein fluorescence. The enzyme has a fluorescence excitation maximum at 280 nm and an emission maximum at 330 nm due to the presence of tryptophan residues in a hydrophobic environment (figure 5). The dye binding was monitored by measuring the fluorescence quenching of the enzyme (50  $\mu$ g/ml) upon the addition of different concentrations of Cibacron Blue. It can be seen from figure 5, that the dye causes a quenching in the intensity of the fluorescence at 330 nm. These data were used to construct a modified Stern-Volmer plot (figure 5A). A  $K_d$  value of 1.2  $\mu$ M from the slope and an 'n' value of 1 per subunit from the Y-axis intercept were calculated. It can be seen from figure 5B that the increasing concentrations of KCl (0.02–0.4 M) reversed the fluorescence quenching caused by 2.5  $\mu$ M Cibacron Blue.

From a similar fluorescence studies for the Procion Red dye (figure 6), a  $K_d$  value of 1.2  $\mu$ M and an 'n' value of 1 were calculated (inset figure 6). KCl reversed the quenching caused by Procion Red dye also.

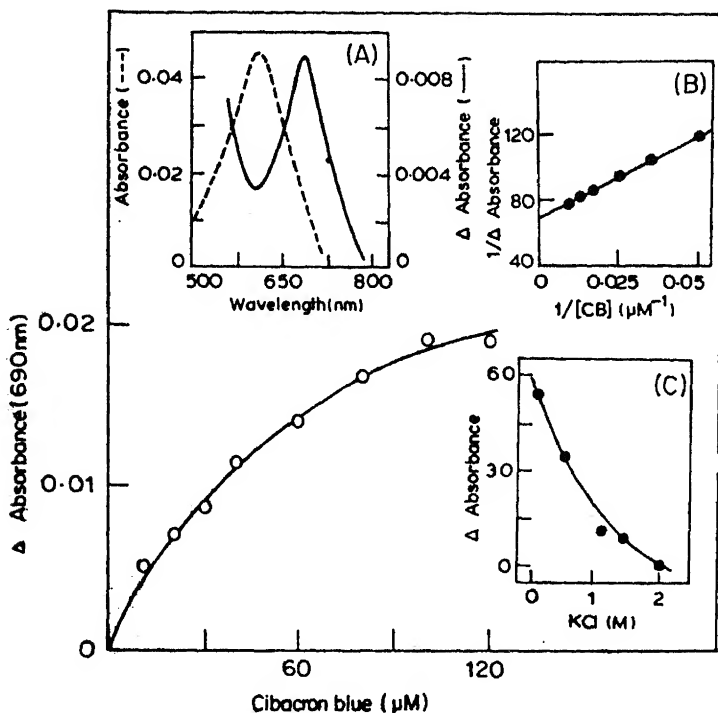
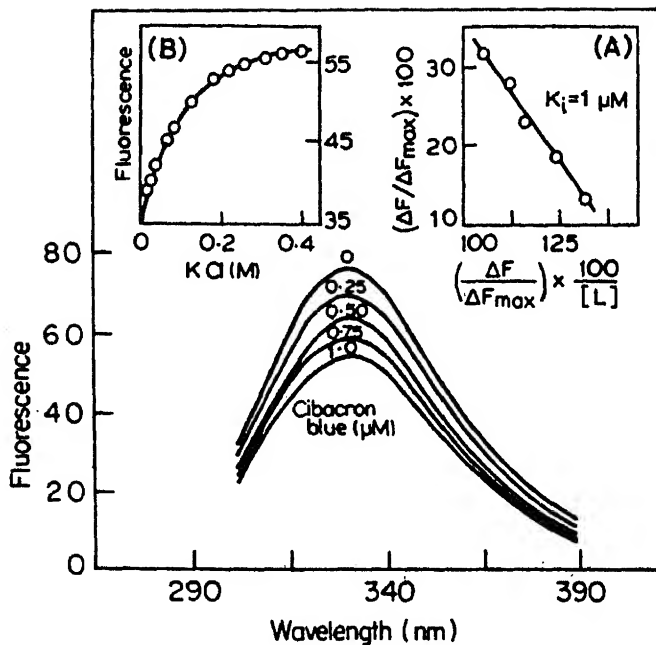


Figure 4. Binding of Cibacron Blue to 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase.

Enzyme (1 mg) in 1 ml of 0.05 M potassium phosphate buffer pH 7.2 containing 0.3 mM EDTA was taken in the sample cuvette and 1 ml of the same buffer was taken in the reference cuvette and the base line was recorded from 500–800 nm. Equal amounts of Cibacron Blue in the concentration range (10–120  $\mu$ M) were added to both the cuvettes and the difference spectra were recorded. The absorbance change at 690 nm was plotted against Cibacron Blue concentration. The inset (A) shows the spectrum (---) of Cibacron Blue and (—) shows the difference spectrum when the enzyme was added to the dye solution. The inset (B) shows the double reciprocal plot for this data. The inset (C) shows the reversal of quenching of enzyme-dye difference spectrum by KCl.

## Discussion

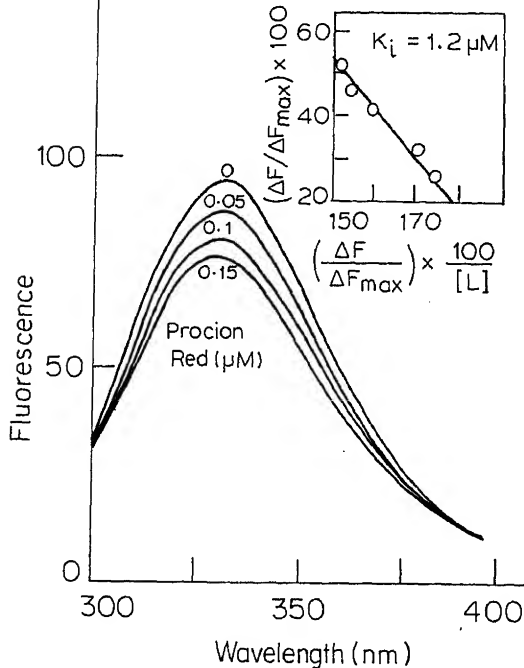
The sulphonated polyaromatic chlorotriazine dye, Cibacron Blue F3G-A has been extensively used for the purification of dehydrogenases and initially it was used as a probe to identify the nucleotide binding domains (Rossmann *et al.*, 1974; Thompson *et al.*, 1975; Thompson and Stellwagen, 1976; Apps and Gleed, 1976; Stellwagen, 1977; Ashton and Polyá, 1978; Lepo *et al.*, 1979). But later work indicated that it was



**Figure 5.** Quenching of the fluorescence of the enzyme by Cibacron Blue F3G-A.

To the enzyme (50  $\mu\text{g/ml}$ ) in 0.05 M potassium phosphate buffer containing 0.3 mM EDTA, different concentrations of Cibacron Blue (0.25–1  $\mu\text{M}$ ) were added and the fluorescence emission spectra were recorded. The inset (A) shows the Stern-Volmer plot for the data. The inset (B) shows the reversal of fluorescence quenching by KCl.

examination of the interaction of the dye with dihydrofolate reductase (Chambers and Dunlap, 1979; Subramanian and Kaufman, 1980) and serine hydroxymethyltransferase (Ramesh and Appaji Rao, 1980) and these studies revealed that the dye binding was unique in each case. The *bi-bi* ping-pong mechanism for the reaction catalyzed by 5,10- $\text{CH}_2\text{-H}_4$  folate reductase with NADPH as the first substrate to add and 5- $\text{CH}_3\text{-H}_4$  folate as the last product to leave the enzyme (Varalakshmi *et al.*, 1983), enabled the prediction that the dye would be a competitive inhibitor with respect to both 5- $\text{CH}_3\text{-H}_4$  folate and NADPH. The results of kinetic analysis for the inhibition by the dye (figures 1 and 2) are in accordance with this prediction. Further evidence to suggest that the dye was binding to the free form of the enzyme was the characteristic difference spectrum of enzyme-dye complex (figure 4A). However, the spectrum was qualitatively different from those obtained with other protein-Cibacron Blue complexes. The peak position in this case was at 690 nm, when compared to 700 nm in chicken liver dihydrofolate reductase (Subramanian and Kaufman, 1980), 645 nm in *L. casei* dihydrofolate reductase (Chambers and Dunlap, 1979) and 670 nm in monkey liver serine hydroxymethyltransferase (Ramesh and Appaji Rao, 1980). The red shift indicates that the dye was probably binding in a hydrophobic region of the molecule. The position of spectral troughs were also different in all the cases. These distinct difference spectra highlight the uniqueness in the dye binding



**Figure 6.** Fluorescence quenching by Procion Red dye.

To the enzyme (50  $\mu\text{g/ml}$ ) in 0.05 M potassium phosphate buffer pH 7.2 containing 0.3 mM EDTA, different concentrations of Procion Red dye were added and the fluorescence emission spectra were recorded. The inset shows the Stern-Volmer plot for this data.

Cibacron Blue closely resembles the dye difference spectrum in the presence of high salt which is called ionic spectrum (Subramanian, 1982). This ionic effect is caused by the shielding of sulfonate groups by the high concentration of sodium ions. This observation indicates that the dye might also be binding in an electrostatic manner to the enzyme.

The binding of the dye to the enzyme was confirmed by the quenching of the intrinsic protein fluorescence and a  $K_d$  value of 1.2  $\mu\text{M}$  was calculated (figure 5A). The similarity in the  $K_i$  values (1.2 and 0.9  $\mu\text{M}$ ) determined from the kinetic experiments (figures 1 and 2) and  $K_d$  value (1.2  $\mu\text{M}$ ) obtained by fluorescence studies (figure 5A) suggested that these probes were probably measuring a similar binding site on the enzyme.

The  $K_d$  value obtained by a double reciprocal plot of the spectroscopic data (figure 4B) was 14  $\mu\text{M}$  compared to the values in the range of 0.9–1.2  $\mu\text{M}$  obtained by other methods. The  $K_d$  value calculated for the same set of data by the method of Thompson and Stellwagen (1976) was 6  $\mu\text{M}$ . Similar discrepancy was observed in the data of Chambers and Dunlap (1979). A recalculation of the constants from the data of Chambers and Dunlap (1979) for the binding of Cibacron Blue to dihydrofolate reductase by a double reciprocal plot analysis was 5  $\mu\text{M}$  and the  $K_d$  value obtained by using the method of Thompson and Stellwagen (1976), was 0.13  $\mu\text{M}$  indicating the inherent difficulties in the analysis of spectroscopic data for obtaining the  $K_d$  values for the binding of this dye to proteins.

The competitive inhibition (figures 1-5), almost identical  $K_i$  and  $K_d$  values (figures 5 and 6) and the reversal of spectral changes by NADPH and 5-CH<sub>3</sub>-H<sub>4</sub> folate strongly suggest that Cibacron Blue and Procion Red are probably interacting at the active site of the reductase. However, there was no enhancement in the reversal when NADPH and 5-CH<sub>3</sub>-H<sub>4</sub> folate were added together suggesting that the dye, NADPH and 5-CH<sub>3</sub>-H<sub>4</sub> folate were probably binding at the same domain in the enzyme but not to identical residues. The bulky aromatic groups in the dye and its structural similarity with both the substrates might direct the dye to the hydrophobic region of the active site of 5,10-CH<sub>2</sub>-H<sub>4</sub> folate reductase and binding could be further strengthened by electrostatic interaction between the charged groups of the dye and the amino acid residues of the enzyme.

## Acknowledgements

We are grateful to the Department of Science and Technology, New Delhi, for financial support.

## References

- Apps, D. K. and Gleed, C. D. (1976) *Biochem. J.*, **159**, 441.  
 Ashton, A. R. and Polya, G. M. (1978) *Biochem. J.*, **175**, 501.  
 Bornmann, L. and Hess, B. (1977) *Z. Naturforsch.*, **C32**, 756.  
 Chambers, B. B. and Dunlap, R. B. (1979) *J. Biol. Chem.*, **254**, 6515.  
 Daubner, S. C. and Matthews, R. G. (1982) *J. Biol. Chem.*, **257**, 140.  
 Jacobsberg, L. B., Kantrowitz, E. R. and Lipscomb, W. N. (1975) *J. Biol. Chem.*, **250**, 9238.  
 Krakow, J. S. (1965) *Biochim. Biophys. Acta*, **95**, 532.  
 Lehrer, S. S. (1971) *Biochemistry*, **10**, 3254.  
 Lepo, J. E., Stacey, G., Wyss, O. and Tabita, F. R. (1979) *Biochim. Biophys. Acta*, **568**, 428.  
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.  
 Matthews, R. G. and Haywood, B. J. (1979) *Biochemistry*, **18**, 4845.  
 Ramesh, K. S. and Appaji Rao, N. (1980) *Biochem. J.*, **187**, 249.  
 Rossmann, M. G., Moras, D. and Olsen, K. W. (1974) *Nature (London)*, **250**, 194.  
 Stellwagen, E. (1977) *Acc. Chem. Res.*, **10**, 92.  
 Subramanian, S. (1982) *Arch. Biochem. Biophys.*, **216**, 116.  
 Subramanian, S. and Kaufman, B. T. (1980) *J. Biol. Chem.*, **255**, 10587.  
 Thompson, S. T., Cass, K. H. and Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 669.  
 Thompson, S. T. and Stellwagen, E. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 361.  
 Varalakshmi, K., Savithri, H. S. and Appaji Rao, N. (1983) *J. Biosci.*, **5**, 287.

# Multisubstrate specific amylase from mushroom *Termitomyces clypeatus*

ANIL K. GHOSH and SUBHABRATA SENGUPTA

Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Calcutta 700 032, India

**Abstract.** An amylase was purified from the culture filtrate of *Termitomyces clypeatus* by ammonium sulphate precipitation, DEAE-Sephadex chromatography and gel filtration on Bio-Gel P-200 column. The electrophoretically homogeneous preparation also exhibited hydrolytic activity (in a decreasing order) on amylose, xylan, amylopectin, glycogen, arabinogalactan and arabinoxylan. The enzyme had characteristically endo-hydrolytic activity on all the substrates tested and no xylose, glucose, arabinose or glucuronic acid could be detected even after prolonged enzymatic digestion of the polysaccharides.

Interestingly the enzyme had similar pH optima (5.5), temperature optima (55°C), pH stability (pH 3–10) and thermal denaturation kinetics when acted on both starch and xylan (larch wood).  $K_m$  values were found to be 2.63 mg/ml for amylase and 6.25 mg/ml for xylanase activity. Hill's plot also indicated that the enzyme contained a single active site for both activities.  $Hg^{2+}$  was found to be most potent inhibitor.  $Ca^{2+}$ , a common activator for amylase activity, appeared to be an inhibitor for this enzyme. Thus it appeared that the enzyme had multisubstrate specificity acting as  $\alpha$ -amylase on starch and also acting as xylanase on side chain oligosaccharides of xylan containing  $\alpha$ -linked sugars.

**Keywords.** Amylase; xylanase; polysaccharidase; *Termitomyces clypeatus*.

## Introduction

Endo-glycosidases, particularly those hydrolysing polysaccharides of biomass into fermentable sugars, have much use in commerce. Different amylases, xylanases (hemicellulases) and cellulases from various microbial sources have largely been screened and studied for their commercial utility. We have reported earlier that mushroom-mycelia under submerged growth elaborates all of these enzymes in good titre (Ghosh and Sengupta, 1982). An interesting endo- $\beta$  (1 $\rightarrow$ 4) xylanase, constitutively produced by mushroom *Termitomyces clypeatus* in dextrin medium, was purified to homogeneity (Ghosh *et al.*, 1980). The enzyme was found to be of high molecular weight ( $M_r$ ) ( $\sim$ 90,000) compared to other reported xylanases (Dekker and Richards, 1976) and also contained hydrolytic activity for amylopectin, arabinoxylan and arabinogalactan. It is to be mentioned that xylan is a complex molecule containing both  $\beta$ -linked xylosidic backbone and  $\alpha$ -glycosidically linked glucose, arabinose and glucuronic acid in branch chains. Perhaps for this reason purified xylanases from various sources has been shown to contain carboxymethyl cellulase, amylase (Comtat *et al.*, 1975) and carboxymethyl cellulase, cellulase and amylase activities (Takahashi and Katsumi, 1979). However it was never identified whether origin of all these activities was because of contamination or lied on the same enzyme molecule.

The present paper describes purification of an extra cellular amylase from the

purification steps and finally in the purified amylase molecule.

## Materials and methods

Synthetic medium for the growth of *T. clypeatus* (Heim) has been described earlier (Ghosh *et al.*, 1980). For the production of enzyme, submerged fermentation was continued at  $30 \pm 1^\circ\text{C}$  for 7 days in a medium containing (% w/v) dextrin, 4;  $\text{NH}_4\text{H}_2\text{PO}_4$ , 2.463;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.037;  $\text{KH}_2\text{PO}_4$ , 0.087;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05;  $\text{H}_3\text{BO}_3$ , 0.057;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.025;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.0036;  $\text{NaMoO}_4 \cdot 4\text{H}_2\text{O}$ , 0.0032;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03; at pH 3.0. Xylan (1,4- $\beta$ -linked) from larch wood, yeast mannan, DEAE-Sephadex (A-50), methyl- $\alpha$ -D-xylopyranoside, methyl- $\beta$ -D-xylopyranoside, amylopectin azure,  $\alpha$ -methyl-D-mannopyranoside, 1-O-methyl- $\alpha$ -D-glucopyranoside, 1-O-methyl- $\beta$ -D-glucopyranoside, arabinogalactan, carboxymethyl cellulose (low viscosity), cellulose, dextran (*M*, 66,900), amylose type III polygalacturonic acid grade III, glycogen type II and sucrose were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Arabinoxylan (arabinose, 34.1% and xylose, 65.9%) was a gift from Dr. G. B. Fincher, Brewing Industry Research Foundation, England. Bio-Gel P-200 (75–150  $\mu\text{m}$ ) was the product of Bio-Rad Laboratories, Richmond, California, USA.

Other chemicals used were of chemically pure quality or better.

### Assay of enzyme activity

This was carried out by measuring the amount of liberated reducing sugar according to the method of Nelson (1944) as modified by Somogyi (1952) and also described earlier (Ghosh *et al.*, 1980). The assay mixture contained 0.02 ml of culture filtrate or enzyme solution, 0.2 ml of xylan or starch (soluble) suspension or solution (10 mg/ml in 0.1 M acetate buffer, pH 5.0) and 0.18 ml of the same buffer. Incubation was carried out for 30 min at  $40^\circ\text{C}$  and stopped by adding 0.4 ml of alkaline copper reagent. Readings were expressed in terms of glucose or xylose equivalents. One unit of enzyme activity was expressed as the amount of enzyme protein which produced one  $\mu\text{mol}$  of D-glucose or D-xylose per min under the assay condition.

### Protein determination

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard or by measuring absorbance at 280 nm during column elution.

### Polyacrylamide gel electrophoresis (PAGE)

It was carried out in glycine-Tris buffer at pH 9.5 using 7.5% acrylamide (Gabriel, 1971). A constant current of 2.5 mA per gel (7.0 cm) was applied for 3 h at  $25^\circ\text{C}$ . Gels were stained with Coomassie brilliant blue for 12 h and destained with methanol/acetic acid/water.

according to Weber *et al.* (1972). Protein standards (Bio-Rad): lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), BSA (66,200) and phosphorylase B (92,500) or enzyme were dissolved in 1.25% SDS buffer, heated on a boiling water bath and electrophoresed. A constant current of 8 mA per gel (8.0 cm) was applied for 6 h at 25°C.

### *Purification of the enzyme*

This was carried out at 4°C unless otherwise specified using the following steps.

*Ammonium sulphate precipitation:* The filtered broth (1660 ml) was brought to pH 6.5 with slow additions of  $K_2HPO_4$ , kept overnight and centrifuged at 50,000 *g* for 30 min. The supernatant was then brought to 80% saturation with  $(NH_4)_2SO_4$ , kept overnight and centrifuged at 105,000 *g* for 60 min. The precipitate was dissolved in 16 ml of 0.01 M acetate buffer (pH 5.0), dialysed against the same buffer, and applied onto a DEAE-sephadex column.

*Chromatography on DEAE-Sephadex (A-50):* The column (4.6 × 27.8 cm) was equilibrated with 0.01 M acetate buffer (pH 5.0). The enzyme solution (15.5 ml) was applied to the column and eluted with the same buffer at a flow rate of 40 ml/h. After passing 4 bed volumes of the buffer (1300 ml), a 0–0.8 M linear NaCl gradient was used.

*Chromatography on Bio-Gel P-200:* The column (3.0 × 30 cm) was equilibrated with 0.1 M acetate buffer (pH 5.0). The enzyme fraction (116–141) eluted with NaCl gradient from the DEAE-Sephadex column was lyophilized to approximately 2.16 ml, dialysed against the same buffer and applied to the column. The enzyme was eluted (3.1 ml/tube) with the same buffer at a flow rate of 5 ml/h. The fraction (30–40) showing activity was used as enzyme source for further studies.

### *Studies on the properties of the purified enzyme*

*Optimum temperature:* Both of the enzyme activities were tested at temperatures between 10–80°C for the determination of optimum enzyme activity.

*Thermal stability:* Enzyme solution (2.6 µg of protein for xylanase assay and 0.52 µg of protein for amylase assay) was kept at pH 5.0 for 0–240 min at 60°C and 0–30 min at 65°C following measurement of residual enzyme activities left.

*Effect of pH on activity:* Universal buffers (citrate/phosphate/borate/barbiturate) of pH between 2.5–10.0 were used in the incubation mixture to determine optimum



at 37°C. Residual activities of these preincubated samples were measured separately using either starch or xylan as substrates.

*Effect of some metal ions and inhibitors:* Enzyme activities were measured in the presence or absence of the test compounds using acetate buffer (0.1 M, pH 5.0).

*Activity towards different substrates:* Assay mixtures containing different carbohydrates (5 mg/ml) were incubated separately for 30 min in 0.1 M acetate buffer, pH 5.0, with 1.0 µg of enzyme.

*Effect of substrate concentration on enzyme activity:* Variable amounts of xylan (1.0–45 mg) and starch soluble (0.2–5.0 mg) in acetate buffer (0.1 M, pH 5.0) were incubated with 2 µg of enzyme during assay of xylanase activity and 0.52 µg of enzyme during assay of amylase activity.

#### *Mode of action on different xylodextrins*

Several xylodextrins were prepared according to Brown and Anderson (1971, 1972). The xylodextrins ( $X_2$ – $X_8$ ) thus prepared were then further purified by passing through a column (1.8 × 33.5 cm) of Sephadex G-25 using water as the eluent and then compared with authentic samples obtained from Prof. G. O. Aspinall, Department of Chemistry, York University, Ontario, Canada. Enzymatic action on xylan, starch, arabinoxylan, arabinogalactan, glucose, xylose and xylodextrins ( $X_2$ – $X_8$ ) were studied separately. Incubation mixtures containing different saccharides were made in 2.0 ml of 0.1 M, pH 5.0 acetate buffer and 52 µg of enzyme and incubated at 40°C for 17 h and 41 h, respectively. The reactions were stopped with the addition of 2 ml of absolute alcohol, shaken vigorously and centrifuged. The supernatants were then deionised and taken out for analysis by thin-layer chromatography (TLC). Plates were made using Kieselguhr buffered with 0.02 M acetate buffer, pH 5.0. The mobile phase used were ethyl acetate:isopropanol:water (75:49:26) for lower dextrins  $dp = 1-3$  and (63:54:33) for higher dextrins  $dp = 4-8$ . Sugars of varied chain lengths were detected on the TLC plate using anisaldehyde reagent (Brown and Anderson, 1971). Dextrins ( $dp > 3$ ) were compared with authentic xylodextrins only. Side by side blank experiments using boiled enzyme solution was also made. Both quantitative and qualitative measurement were made visually with the intensity of the colour produced on the chromatogram. Higher dextrins ( $dp > 8$ ) were not available for further studies.

## **Results**

Hydrolytic activity towards soluble starch and larch wood xylan was measured as a function of growth periods (figure 1). Activity towards hydrolysing both xylan (xylanase) and starch (amylase) remained almost same during early periods of growth. From 5th day onwards, amylase activity increased significantly while xylanase activity was not found to be increasing similarly. Though from 10th day

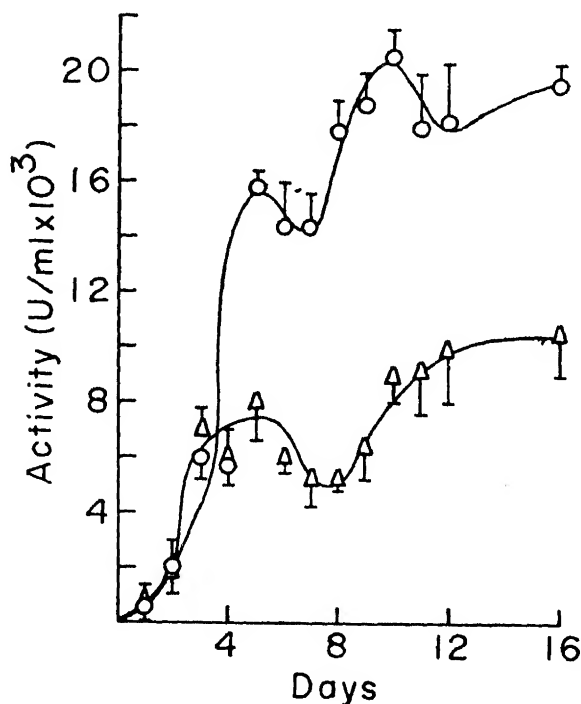


Figure 1. Production of extracellular xylanase and amylase during submerged growth of mushroom *T. clypeatus*.

Activities (O, amylase activity and  $\Delta$ , xylanase activity) measured represent mean  $\pm$  standard error of mean were determined as detailed in 'materials and methods'.

onwards about 1.5-fold increase in xylanase activity was detected but amylase activity was almost double or more than double than xylanase activity from 5th day onwards.

The enzyme was purified from the culture filtrate following different steps (table 1).

During purification of the enzyme it was observed that direct addition of ammonium sulphate to the culture filtrate caused significant loss in enzyme activity. This loss could be avoided by prior adjustment of pH of the culture filtrate to 6.5 with the addition of solid  $K_2HPO_4$ . This treatment caused a precipitation with no such loss in enzyme activity present in the supernatant ( $\sim 90\%$  of total activity). Unfortunately after  $(NH_4)_2SO_4$  precipitation of the buffered culture filtrate about 10–30% of the total activity could only be obtained with 2–6 fold purification.

The  $(NH_4)_2SO_4$  precipitated fraction was chromatographed on a DEAE-Sephadex (A-50) column. During washing with buffer a small fraction of enzyme activity was eluted in between fractions 51–54. Later, elution with NaCl gradient, majority of the enzyme activity (fractions 116–141, 156 ml) containing both xylanase and amylase were eluted in the same peak. At this point the enzyme was further purified 3.5-fold with practically no loss in recovery. Next the enzyme was purified

Table 1. Purification of enzyme from the culture filtrate of *T. clypeatus*.

Enzyme sample	Protein (mg)	Total activity (U $\times 10^{-3}$ )		Specific activity (U/mg of protein)		Recovery yield (%)		Purification (-fold)	
		Xylanase	Amylase	Xylanase	Amylase	Xylanase	Amylase	Xylanase	Amylase
Culture filtrate (1660 ml)	1344.6	5.902	4.55	4.39	3.38	100	100	1	1
Buffered culture filtrate (1650 ml)	808.5	5.42	4.28	6.71	5.29	91.95	94.07	1.53	1.57
Ammonium sulphate (0-80%) saturation (16 ml)	65.92	0.626	1.42	9.5	21.54	10.61	31.21	2.16	6.37
DEAE-Sephadex (A-50) fractions 116-141 lyophilised to 12.6 ml	20.03	0.56	2.33	27.96	116.3	9.49	51.21	6.37	34.41
Bio-Gel P-200 fractions 27-39 lyophilised to 10 ml	6.8	0.276	1.77	40.6	260.3	4.68	38.90	9.25	77.01

recovery. Surprisingly, the same fraction contained 77 fold increase in amylase activity with 39% recovery. The fraction collected from the Bio-Gel P-200 column was found to be homogeneous upon PAGE and on SDS-PAGE. No mobility of the enzyme protein was observed at lower pH values.  $M_r$  of the enzyme protein was estimated to be 85,000 upon SDS-PAGE and found to contain no subunit or similar subunits.

Enzymatic activities were then measured as a function of temperature. Both xylanase and amylase activities were found to reach optimum at 55°C. Activation energy (E) for xylanase and amylase activity were found to be 12.8 KCal and 11.8 KCal, respectively. Preincubation of the enzyme solution for 1 h at 35°C or below did not cause any loss in activity but similar treatment at 60°C cause 40–50% decrease in enzyme activity.

Preincubation of the enzyme solution at 60°C for 0–240 min (figure 2a) or at 64°C for 0–30 min (figure 2b) was made following measurement of residual enzyme activity left. The rate of loss in both amylase and xylanase activities were biphasic either at 60°C or at 64°C. At 60°C about 65% of amylase and 43% of xylanase activities were lost by initial 72 min of preincubation while after 240 min of preincubation about 88% of amylase and 66% of xylanase activities were lost. Similarly at 64°C about 40% of amylase and 18% of xylanase activities were lost by only initial 9 min period of preincubation while after 30 min of preincubation 82% of amylase and 43% of

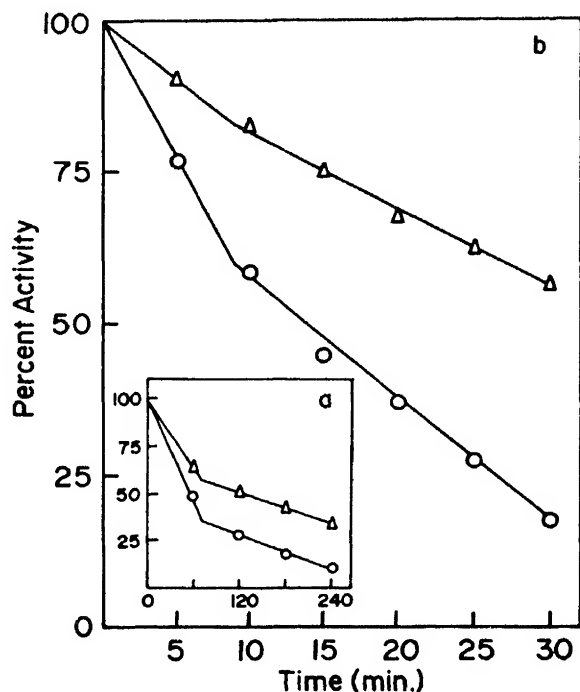


Figure 2. Thermal stability kinetics of the enzyme.

Enzyme solution containing both amylase (O) and xylanase ( $\Delta$ ) activities were pre-incubated either at 60°C (a) or at 64°C (b) for different time periods and the residual activity

xylanase activities were lost. About 40% loss in both xylanase and amylase activities were noticed when the enzyme solution was kept at 30°C, pH 5.0 for several days but about 65% loss in both of the activities were found to be gone by 4 h only after keeping at 40°C (data not shown). The enzyme was sensitive towards freezing and lyophilisation with 15% loss in activity per freezing and thawing cycle.

Optimum pH for both of the activities were found to be 5.5. About 50% decrease in optimum enzyme activity was noticed either at pH 3.0 or at pH 6.5. No activity was left when measured at pH more than 7.0. Stability of the enzyme for both of the activities at different pH values were measured at 40°C. It was noticed that the enzyme was relatively stable over a wide range of pH values (3.0–10.0) at least for 1 h.

The degree of potency in terms of inhibitory effect of all the chemicals tested has been presented in a decreasing order in table 2. Very little, 2.5% and 5.5% of total amylase and xylanase activity respectively could be detected when  $\text{Hg}^{2+}$  was present in the incubation mixture at a level of 2 mM only.  $\text{Ag}^+$  and  $\text{Fe}^{2+}$  also had some inhibitory effect on both of the enzymatic activities. BSA (100  $\mu\text{g}/\text{ml}$ ) and dextran (0–0.45%, w/v) had no effect.

Activity towards different carbohydrates were next studied (table 3). The enzyme

**Table 2.** Effect of some metal ions and inhibitors.

Chemicals	Residual activity (%)	
	Amylase	Xylanase
$\text{Hg}^{2+}$ (2 mM)	2.5	5.5
$\text{Ag}^+$ (2 mM)	52.0	61.5
$\text{Fe}^{2+}$ (2 mM)	64.0	67.5
$\text{Ca}^{2+}$ (20 mM)	75.0	86.0
$\text{Ca}^{2+}$ (100 mM)	10.0	15.5
$\text{Na}_2\text{HPO}_4$ (100 mM)	45.0	50.0
$\text{CH}_2\text{I}\text{COOH}$ (20 mM)	11.5	5.0
EDTA (20 mM)	30.0	17.5

Incubations were made using 2.0  $\mu\text{g}$  of enzyme for measuring xylanase activity and 0.52  $\mu\text{g}$  of enzyme for measuring amylase activity as detailed in 'materials and methods'.

**Table 3.** Enzymatic activity on different substrates.

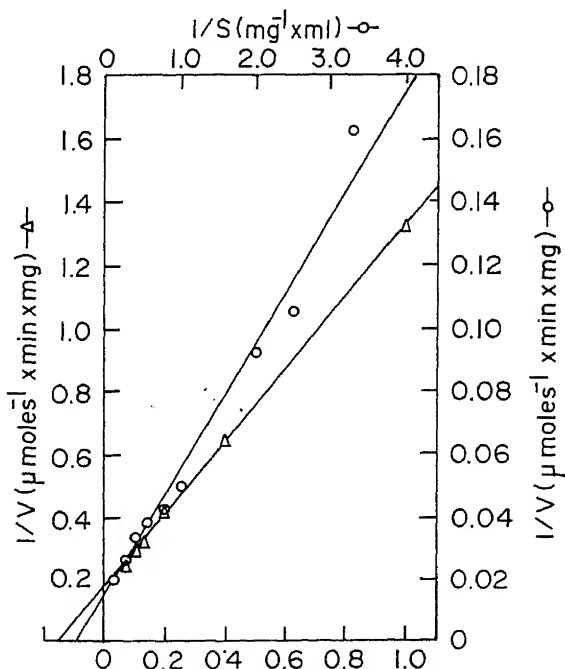
Substrate (5 mg/ml)	Activity ( $\text{U} \times 10^{-1} \pm \text{SEM}$ )
Starch soluble	$17.20 \pm 1.2$
Amylose	$16.37 \pm 0.9$
Amylopectin	$1.33 \pm 0.05$
Glycogen	$1.33 \pm 0.20$
Arabinogalactan	$0.52 \pm 0.02$
Arabinoxylan	$0.62 \pm 0.02$
Xylan	$3.20 \pm 0.18$

Enzymatic action towards different polysaccharides were tested separately and the activities were expressed as glucose or xylose equivalents produced

had no  $\alpha$ - or  $\beta$ -xylosidic or glucosidic or  $\alpha$ -mannosidic activity. Cellulose, CM-cellulose, sucrose, mannan, dextran and polygalacturonic acid were not at all attacked by the enzyme. Among the carbohydrates tested, starch (soluble) and amylose were primarily hydrolysed by the enzymatic action. Hydrolysing activity towards xylan was less than one fifth of the activity towards starch (soluble).

Mode of action of the enzyme towards xylan or starch or xylooligosaccharides (only oligosaccharides available) were carried out by incubating respective substrates in the presence of the enzyme and the products obtained were analysed by TLC as described earlier. Incubation of the enzyme with arabinoxylan or arabinogalactan could produce very minute quantity of xylose or galactose. Presence of arabinose in the enzyme-hydrolysed incubation mixture was never noticed. Incubation containing xylan or starch with the enzyme, produced dextrans of varied chain length ( $dp \geq 3$ ) among which xylooligosaccharide ( $X_4$ ) was appeared to be the principal product. Xylobiose ( $X_2$ ) and xylooligosaccharide ( $X_3$ ) remained almost unattacked by the enzyme. Xylooligosaccharide ( $X_7$ ) and xylooligosaccharide ( $X_8$ ) were readily attacked by the enzyme producing mainly xylooligosaccharide ( $X_4$ ) while xylooligosaccharide ( $X_6$ ) was attacked by the enzyme to make xylobiose ( $X_2$ ) and xylooligosaccharide ( $X_3$ ) together with xylooligosaccharide ( $X_4$ ). Xylopentose ( $X_5$ ) also was attacked by the enzyme to liberate mainly xylooligosaccharide ( $X_4$ ).

Effect of various concentrations of starch and xylan on the enzymatic activities were studied and the corresponding activities measured were analysed on a Lineweaver-Burk plot (figure 3).  $V_m$  values were found to be  $64.52 \mu\text{mol glucose}$



starch/ml for amylase and 6.63 mg of xylan/ml for xylanase. Both amylase and xylanase activities were further analysed by the linear plot of Hill equation. Both of the slopes ( $n=0.94 \approx 1$  using starch and  $n=1.16 \approx 1$  using xylan) had a value of unity.  $S_{1/2}$  values were 2.63 mg of starch/ml and 6.17 mg of xylan/ml.

## Discussion

Increase in amylase activity with respect to increase in xylanase activity during later periods of growth was not clearly understood (figure 1). This may be related to the increase in some inhibitors from 5th day onwards. Increase in amylase activity was similarly noticed during purification of the enzyme (table 1) which might be due to the removal of some inhibitors. Characterisation of this natural inhibitors had not been ruled out by some additional experiments since we had been principally interested in isolating an enzyme with multisubstrate specificity. Loss in enzyme activity during ammonium sulphate precipitation of the unbuffered culture filtrate was very similar to the observation, we have reported earlier (Ghosh *et al.*, 1980). Unfortunately significant quantity ( $\sim 80\%$ ) of xylanase activity was lost in comparison to loss in amylase activity ( $\sim 60\%$ ) by ammonium sulphate precipitation. Elution pattern of the enzyme having both amylase and xylanase activity either from the ion-exchange column or from the gel filtration column were interestingly similar. Physicochemical properties and stability towards pH and temperature for both of the activities were also same and resemble in various respects to non-arabinose liberating endo- $\beta$ -D-xylanases (Dekker and Richards, 1976). No increase in enzymatic activity in terms of either xylanase or amylase was observed in the presence of  $\text{Ca}^{2+}$ . Moreover, both the activities were significantly inhibited when measured in the presence of  $\text{Ca}^{2+}$ . Moreover, both the activities were significantly inhibited when measured in the presence of  $\text{Ca}^{2+}$  (table 2) which is in contrast to common amylases (Thoma *et al.*, 1971). Inhibition of the enzyme activity in the presence of  $\text{PO}_4^{3-}$  was quite similar to the observation we reported earlier (Ghosh *et al.*, 1980). So at this point it may be assumed that the present enzyme was a single enzyme capable of hydrolysing both starch and xylan since the enzyme was electrophoretically homogeneous. The degree of starch hydrolysing activity was approximately 6 times more than xylan hydrolysing activity. Xylan from larch wood is an unbranched chain of xylopyranose residues, with every fifth or sixth residue substituted at  $\text{C}_2$  with a 4-O-methoxy-D-glucuronic acid unit and with a small number of xylopyranose residues substituted at  $\text{C}-3$  with arabinofuranose units (Aspinall and McKay, 1959). Acid hydrolysis of Sigma xylan has been shown to contain xylose, 48–71%; glucose, 10–13%; arabinose, 10–11%; glucuronic acid 8–29% and some unhydrolysed polymer (Frederick *et al.*, 1981). From the Lineweaver-Burk plot,  $K_m$  values for xylanase and amylase were 6.25 mg/ml and 2.63 mg/ml respectively (figure 3) indicating that the enzyme had lesser affinity for attacking xylan. But considering other observations the enzyme may be considered as a xylanase and not a commonly reported amylase (Thoma *et al.*, 1971). The enzyme could cleave  $\alpha$ -1,4 glycosyl linkages present either in xylan or in starch with or without additional capacity for breaking  $\beta$ -1,4 and  $\alpha$ -1,6 linkages. Similar report is also available for a thermostable xylanase purified from a thermo-

pinic acidophilic *Bacillus* Sp. (Uchino and Nakane, 1981). This was further confirmed by analysing the enzyme hydrolysed larch wood xylan by TLC. The predominant hydrolysed products from larch wood xylan were xyloextrins of varied chain lengths ( $dp > 3$ ). No xylose, glucuronic acid, arabinose or glucose were detected in the enzyme hydrolysed mixture.

Glycogen and amylo-pectin were poorly attacked by the enzyme in comparison to starch and amylose. Similarly arabinoxylan and arabinogalactan were poorly attacked with respect to xylan. So the enzyme may be considered as an endoenzyme and possibly had a preference in cleaving less branched polysaccharides. On the basis of all these facts the enzyme may be classified as a non arabinose liberating endo xylanase, as mentioned by Dekker and Richards (1976) cleaving principally  $\alpha, 1 \rightarrow 4$  glycosyl linkages.

## Acknowledgement

The authors are grateful to Professor Bimal K. Bachhawat, ex-director of the institute for his kind interest and valuable suggestions during the progress of the work. Uninterrupted support from Dr. S. C. Pakrashi, is highly appreciated. Thanks are also due to Dr. G. B. Fincher, Brewing Industry Research Foundation, Nutfield, Redhill, Surrey, UK for arabinoxylan and Dr. G. O. Aspinall, Trent University, Ontario, Canada for providing xyloextrins ( $dp$  2-8) as generous gift.

## References

- Aspinall, G. O. and McKay, J. E. (1959) *J. Chem. Soc.*, **1959**, 1059.  
Brown, W. and Anderson, O. (1971) *J. Chromatogr.*, **57**, 255.  
Brown, W. and Anderson, O. (1973) *J. Chromatogr.*, **67**, 163.  
Comtat, J., Ruel, K., Joseleau, J. and Barnoud, F. (1975) *Symp. Enz. hydrolysis of cellulose*, Aulanko, Finland, 12-14 March, p. 351.  
Dekker, R. F. H. and Richards, G. N. (1976) *Adv. Carbohydr. Chem. Biochem.*, **32**, 227.  
Frederick, M. M., Frederick, J. R., Fratzke, A. R. and Reilly, P. J. (1981) *Carbohydr. Res.*, **97**, 87.  
Gabriel, O. (1971) *Methods Enzymol.*, **22**, 565.  
Ghosh, A. K. and Sengupta, S. (1982) *Acta Mycol.*, **18**, 113.  
Ghosh, A. K., Banerjee, P. C. and Sengupta, S. (1980) *Biochim. Biophys. Acta*, **612**, 143.  
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.  
Nelson, N. (1944) *J. Biol. Chem.*, **153**, 375.  
Somogyi, M. (1952) *J. Biol. Chem.*, **195**, 19.  
Takahashi, M. and Katsumi, S. (1979) *J. Ferment. Technol.*, **57**, 434.  
Thoma, J. A., Spradlin, J. E. and Dygert, S. (1971) *Enzymes*, **5**, 115.  
Uchino, F. and Nakane, T. (1981) *Agric. Biol. Chem.*, **45**, 1121.  
Weber, K., Pringle, J. R. and Osborn, M. (1972) *Methods Enzymol.*, **26**, 3.





## Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity

P. SHASHIDHARAN and L. K. RAMACHANDRAN†

Department of Biochemistry, Osmania University, Hyderabad 500 007, India

**Abstract.** The essential role of tyrosine residue(s) of cardiotoxin II in the biological activity of the toxin was evaluated using N-bromosuccinimide. N-bromosuccinimide effected oxidation of the tyrosine residues in cardiotoxin II with enhancement in absorbance at 260 nm. The influence of various solvent media such as acetate-formate buffer (pH 4.0), 0.01 N H<sub>2</sub>SO<sub>4</sub> (pH 2.0) and Tris-HCl buffer (pH 8.5) on oxidation of tyrosine residues was examined. In comparison with 0.01 N H<sub>2</sub>SO<sub>4</sub>, acetate-formate buffer could prevent secondary oxidations as revealed by lower consumption of oxidant, N-bromosuccinimide, to achieve oxidation. In Tris-HCl buffer oxidation of tyrosine did not take place effectively. N-iodosuccinimide caused only limited oxidation as evident from minor increase in absorbance at 260 nm. N-chlorosuccinimide was completely ineffective. Oxidation of cardiotoxin II with 3.75 equivalents of N-bromosuccinimide tyrosine residue led to complete loss of lethal activity. However, the derivative retained the ability to protect bacterial protoplasts from lysis in solutions of low tonicity. Unlike cardiotoxin II oxidized with N-chlorosuccinimide (50 equivalents/mol of toxin) which retained lethal activity as well as the ability to protect protoplasts from lysis, performic acid-oxidized toxin had lost both the activities.

**Keywords.** Cardiotoxin II; *Naja naja*; N-bromosuccinimide oxidation; essential tyrosine; protoplast stabilization.

### Introduction

Cardiotoxins are non-neurotoxic protein constituents of cobra venom. These basic toxins are known for their membrane destabilizing property (Condrea, 1974). Recently we have shown that cardiotoxin II stabilizes bacterial protoplasts against lysis under hypotonic condition (Shashidharan and Ramachandran, 1984).

Chemical modification studies on biologically active proteins are carried out in delineating amino acid side chains which are important for their structure and function. An earlier report of ours had dealt with the side-chain amino groups of cardiotoxin II for biological activity (Shashidharan and Ramachandran, 1985). In this paper observations on modification of tyrosine residues with N-bromosuccinimide (NBS) are reported. Earlier reports (Keung *et al.*, 1975; Carlsson, 1980) of modifications of tyrosine sidechains in cardiotoxins had been achieved using tetranitromethane (TNM). Cardiotoxin II is eminently suited for oxidative modification studies with NBS, because the toxin contains neither tryptophan nor histidine (Srinivasa *et al.*, 1982) whose side chains also are known to be susceptible to modification by the reagent (Witkop, 1961). The usefulness of other oxidizing agents such as N-

Cardiotoxin II was purified as described earlier (Achyuthan *et al.*, 1980); *Micrococcus lysodeikticus* (lyophilised cells) and *p*-chloromercuribenzoate (PCMB) were from Sigma Chemical Co., St. Louis, Missouri, USA; egg-white lysozyme was a gift from Societa Prodotti Antibiotici, Milano; NBS from Koch Light, UK; sodium borohydride from British Drug House, UK; trinitrobenzene sulphonic acid (TNBS) and NCS were gift samples; NIS was recrystallized (dioxane- $\text{CCl}_4$ ) before use and had a m.p.  $201^\circ\text{C}$  (reported  $201^\circ\text{C}$ ); dansyl chloride was from Pierce, USA and polyamide sheets were from Schleicher and Schuell, Federal Republic of Germany. All other chemicals were analytical grade commercial samples.

The measurements of light absorption were done with a Shimadzu spectrophotometer (Model UV-150-02) or a MSE Spectro-Plus (Model MK1A) or a Coleman Junior spectrophotometer (Model 6A).

### *Oxidation of cardiotoxin II with NCS, NBS and NIS*

Appropriate amounts (0.325 mg) of cardiotoxin II were dissolved in 3 ml of acetate-formic acid buffer (0.05 M, pH 4.0), or 0.01 N  $\text{H}_2\text{SO}_4$  or Tris-HCl buffer 0.1 M, pH 8.5. Aliquots (10  $\mu\text{l}$ ) of aqueous solutions of NCS or NBS or ethanolic solutions of NIS were added to samples and mixed well, so as to ultimately secure levels in excess of 1.5 equivalents of oxidant/residue of tyrosine in cardiotoxin II. The increase in absorbance at 260 nm was measured after each addition. Necessary corrections were made for dilution with appropriate blanks run simultaneously. The additions were continued until there was no further increase in absorbance at 260 nm. Based on the content of tyrosine residues in cardiotoxin II (Srinivasa *et al.*, 1982) and the increase in absorbance at 260 nm, the equivalents of NBS required to oxidize one to four tyrosine residues were determined. For further studies unless otherwise mentioned, oxidized derivatives were prepared by adding the requisite amount of oxidizing agent to known amounts of cardiotoxin II and the derivatives desalted on Sephadex G-10 columns, using 0.1 N acetic acid for elution.

Consumption of NBS due to oxidation of formate in acetate-formate buffer was followed by iodometry with buffer of strength of 0.005 M containing 0.011 M NBS. At different time intervals, 5 ml of the mixture was withdrawn, excess KI added and iodine liberated titrated against 0.01 N sodium thiosulphate.

### *Performic acid oxidation*

To 0.1 ml of  $\text{H}_2\text{O}_2$  (30%) was added 0.9 ml of 98%  $\text{HCOOH}$  and the same left at room temperature ( $30^\circ\text{C}$ ) for 1 h, followed by cooling to  $0^\circ\text{C}$ . Cardiotoxin II (1 mg) was treated with 1 ml of the above solution at  $0^\circ\text{C}$ , for 1 h. The sample was then diluted to 10 ml with water and lyophilised. To remove any traces of performic acid remaining, the residue was redissolved in 0.5 ml of distilled water and lyophilised again. This procedure was repeated twice.

Reversion of methionine sulphoxide generated, during the oxidation, back to methionine in the oxidized protein (0.5 mg) was achieved by treatment with 0.1 ml of mercaptoethanol, overnight, at room temperature ( $26\text{--}28^\circ\text{C}$ ). The excess of mer-

### *Estimation of free amino groups*

Free amino groups in cardiotoxin were estimated using the method of Fields (1972). The number of free amino groups in the oxidized derivatives was calculated based on the molar absorption ( $E_M$  for TNP group 12,587) of products of reaction with TNBS. There are 10 free amino groups in cardiotoxin II (Srinivasa *et al.*, 1982).

### *Determination of disulphide bonds*

Estimation of disulphide bonds was carried out by an indirect method. The toxin was reduced and the resulting  $-SH$  groups were estimated using PCMB as described by Boyer (1954).

The reduction of cardiotoxin II and oxidized derivatives was done using sodium borohydride. To appropriate amounts (0.1–0.4 mg) of sample, 0.5 ml of 5% sodium borohydride was added and the tubes stoppered and left at  $37 \pm 1^\circ C$  for 2.5 h, after which excess borohydride was destroyed by the addition of 0.1 ml of 50% acetic acid. The last traces of borohydride were eliminated by wetting the sides of the tube with 2 ml of 0.33 M acetate buffer of pH 4.6, so as to avoid interference during estimation of  $-SH$  groups using PCMB. After 5 min the required amount (1.5–2.0 mol/mol of  $-SH$  group) of PCMB was added and mixed well. After 15 min at room temperature the increase in absorbance at 255 nm was recorded against appropriate blanks. The number of  $-SH$  groups was calculated using the known molar absorbance ( $E_{M255\text{ nm } 6200}$ ).

### *Dansylation*

Dansylation of cardiotoxin and derivatives was carried out according to Hartley (1970).

### *Toxicity*

The toxicity of the oxidized products (NBS and NCS oxidized) was assayed by injecting weights of material corresponding to one to ten  $LD_{50}$  doses of native cardiotoxin II, in 0.5 ml of 0.9% saline, intraperitoneally, into Swiss albino mice. The animals were kept under observation for a period of 72 h thereafter.

*Assay of stabilization of bacterial protoplasts:* The assay was carried out as described earlier (Shashidharan and Ramachandran, 1984).

## **Results**

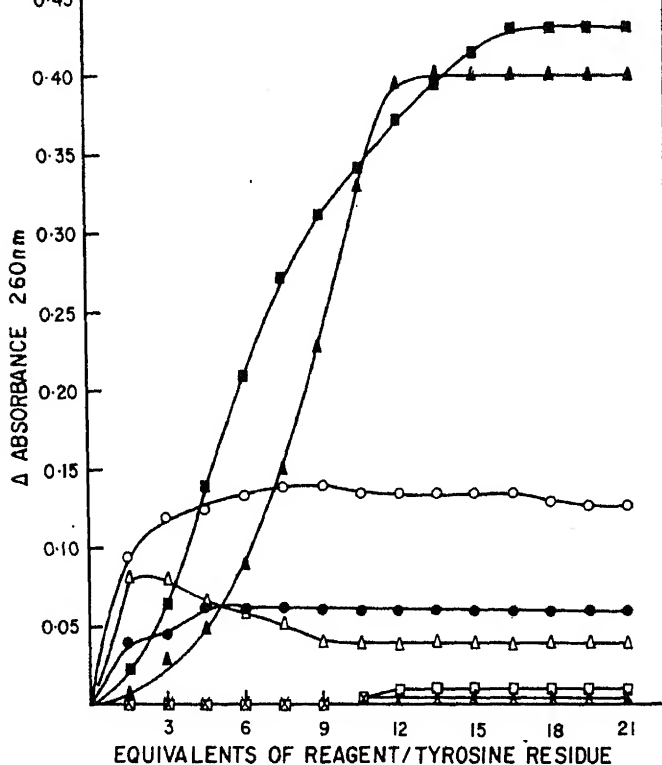


Figure 1. Oxidation of cardiotoxin II (0.0158  $\mu\text{mol/ml}$ ) under varying conditions with increasing concentrations of the reagents: with NBS: (▲), pH 2.0; (■), pH 4.0; (●), pH 8.5; with NCS: (×), pH 4.0; (□), pH 8.5; with NIS: (○), pH 4.0; (△), pH 8.5.

at 260 nm with increments in amount of reagent used is highest with NBS in acidic conditions. Amounts of reagent (NBS) consumed for different degrees of oxidation with acetate-formic acid buffer and 0.01 N  $\text{H}_2\text{SO}_4$  as media are given in table 1. UV spectra of the toxin oxidized with NBS at pH 4.0 are shown in figure 2, while spectra

Table 1. Oxidation of tyrosine residues of cardiotoxin II (0.0158  $\mu\text{mol/ml}$ ) in acetate-formate buffer and 0.01 N  $\text{H}_2\text{SO}_4$ .

Tyrosine oxidized no. of residues	Solvent			
	Acetate-formate buffer pH 4.0		0.01 N $\text{H}_2\text{SO}_4$	
	Absorbance (260 nm)	Eqs. of NBS per tyrosine residue	Absorbance (260 nm)	Eqs. of NBS per tyrosine residue
1	0.107	3.75	0.10	6.30
2	0.215	6.25	0.20	8.40
3	0.322	9.00	0.30	10.05
4	0.430	16.87	0.40	13.50

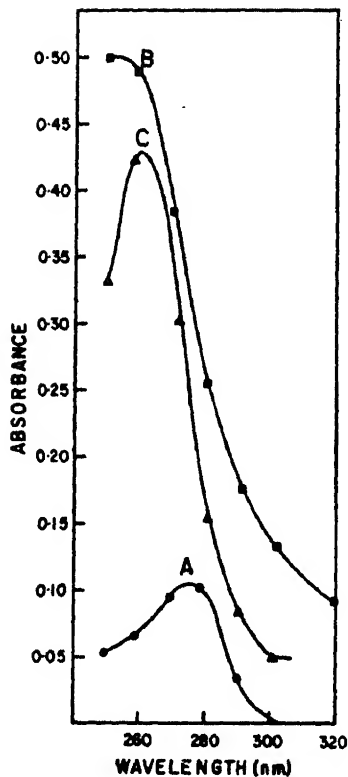


Figure 2. UV spectra of native and fully NBS-oxidized cardiotoxin II ( $0.0158 \mu\text{mol/ml}$ ). (A), native cardiotoxin II; (B), cardiotoxin II oxidized in acetate-formate buffer  $0.05 \text{ M}$ , pH 4.0; (C), cardiotoxin II oxidized in  $0.01 \text{ N H}_2\text{SO}_4$ .

obtained at pH 8.5 and for oxidations with NCS and NIS in different solvent media are not shown.

The molar absorbance ( $E_{M260}$ ) of an oxidized tyrosine residue in cardiotoxin was found to be 7288 in acetate-formate buffer and 6780 in  $0.01 \text{ H}_2\text{SO}_4$ . Reported values in the range 10,000–11,000 for dibromodienone spirolactone derivative of tyrosine are taken as a basis for calculations (Ramachandran and Witkop, 1967). The spectrophotometric data would correspond to oxidation of 2.65–2.85 mol of tyrosine/mol of toxin.

#### *Destruction of NBS by solvent system*

Within 20 min after addition of the reagent (NBS) more than 50% of the reagent was destroyed in  $0.01 \text{ M}$  acetate-formate buffer (pH 4.0) of one-tenth the molarity used in routine experiments in which consumption of reagent in oxidation of formate would be more rapid. The rate of destruction of NBS in original buffer could not be follo-

Table 2 presents data on number of free amino groups in cardiotoxin II and oxidized derivatives.

**Table 2.** The free amino groups in cardiotoxin II and oxidized cardiotoxin II as determined with TNBS.

Sample	Molar absorbance* ( $E_M$ 420 nm)	No. of free amino groups
Native cardiotoxin II	1,25,874	10.00
Cardiotoxin II with one Tyr oxidized	1,23,786	9.80
Cardiotoxin II with two Tyr oxidized	1,20,145	9.50
Cardiotoxin II with three Tyr oxidized	1,17,718	9.18
Cardiotoxin II with four Tyr oxidized	87,378	6.94
Cardiotoxin II with performic acid	1,25,600	9.9

\*Concentration of toxin and the derivatives in the range 0.412–0.429  $\times 10^{-5}$  M were used.

### *Intactness of disulphide bonds*

Table 3 provides data on the integrity of disulphide bonds. No cleavage of disulphide bonds occurs on oxidation at NBS levels of 3.75 eqs/tyrosine residue (S. No. 2). With excess of reagent *i.e.*, 6.25, 9.0 and 16.87 eqs of NBS/tyrosine residue (S. Nos. 3, 4, 5), 0.8, 1.51 and 2.05 disulphide bonds are, respectively, cleaved oxidatively per mol of cardiotoxin.

**Table 3.** Number of—SH groups generated on reduction of cardiotoxin II and its NBS-oxidized derivatives with sodium borohydride.

Sample	Conc. of sample used for—SH measurement (M)	Molar absor- bance change in the PCMB reaction ( $E_M$ 255 nm)	No. of—SH groups gene- rated on borohydride reduction
Native cardiotoxin II	$0.296 \times 10^{-5}$	50,000	8.06
Cardiotoxin II with one Tyr oxidized	$0.425 \times 10^{-5}$	48,717	7.85
Cardiotoxin II with two Tyr oxidized	$0.596 \times 10^{-5}$	39,705	6.40
Cardiotoxin II with three Tyr oxidized	$0.568 \times 10^{-5}$	31,092	5.01
Cardiotoxin II with four Tyr oxidized	$0.948 \times 10^{-5}$	24,369	3.90
Cardiotoxin II oxidized with performic acid	$0.948 \times 10^{-5}$	0	0

### *Cleavage of peptide bonds*

The release of new terminal amino groups as monitored by dansylation of oxidized products showed that there was no detectable new amino terminal residues released on oxidation of cardiotoxin II with NBS. Dansylation and hydrolysis did not reveal

however present as expected in the hydrolyzate of dansylated native protein. But performic acid-oxidized cardiotoxin on further oxidation with NBS (16.87 eqs/tyrosine residue) contained 3 new amino terminal residues (not identified), indicating peptide bond cleavage under such conditions.

### *Toxicity of oxidized toxin*

The derivative obtained on oxidation with 3.75 eqs of NBS/tyrosine residue was found to be completely lacking in lethal potency. The derivative was inactive at a level corresponding to 10 LD<sub>50</sub> doses of native cardiotoxin II. That oxidation of cardiotoxin II at a level of 8 eqs NCS/mol of cardiotoxin II did not result in any inactivation of the lethal potency which was revealed by its ability to induce death in mice at one LD<sub>50</sub> dose. A sample oxidized with 50 eqs NCS/mol of cardiotoxin was also found to cause death in mice but with amounts corresponding to 10 LD<sub>50</sub> doses of native cardiotoxin II. Performic acid oxidized toxin and the sample in which methionine sulphoxides formed on performic acid oxidation were reverted to methionine lacked lethality even at such high dose levels.

### *Ability of oxidized derivatives to stabilize bacterial protoplasts from lysis*

The lysis of bacterial cells in the presence of various derivatives as followed by decrease in turbidity is illustrated in figure 3. The effect of NBS-oxidized derivative on bacterial protoplast stabilization and the ability to cause the initial expected increase in absorption of the bacterial cell suspension are shown in figure 4. The derivatives with one or two tyrosine residues oxidized, and the derivative from oxidation with 50 eqs NCS/mol of cardiotoxin were as efficient as native toxin. With 3 and 4 tyrosine residues of cardiotoxin oxidized, a decrease of 7 and 50% in stabilization ability was observed. Performic acid oxidized cardiotoxin II and the same derivative in which the methionine was regenerated were not at all active as stabilizing agents. On comparison of the ability of the derivatives to enhance absorbance of bacterial cell suspensions, it was found that loss of 1 or 2 tyrosine residues caused a fall of 36% in this ability, while loss of 3 and 4 tyrosines, caused a fall of 50 and 87% respectively. On the other hand the NCS-oxidized sample retained 100% of this ability, and the performic acid-oxidized toxin and the sample in which the methionine sulphoxide formed on performic acid oxidation was reverted back to methionine which exhibited a 100% loss of this ability.

## **Discussion**

The NBS-oxidation of tyrosine side chains in cardiotoxin II has been examined in the present study in the medium 0.05 M acetate-formic acid buffer of pH 4.0, a buffer credited with the ability to afford protection to protein from secondary oxidations (Patchornik *et al.*, 1960). NBS decomposes to CO<sub>2</sub>, hydrobromic acid and succinimide in the above medium instantaneously (Barakat *et al.*, 1955). For reasons which are therefore evident, no net consumption for oxidation of cardiotoxin was observed.



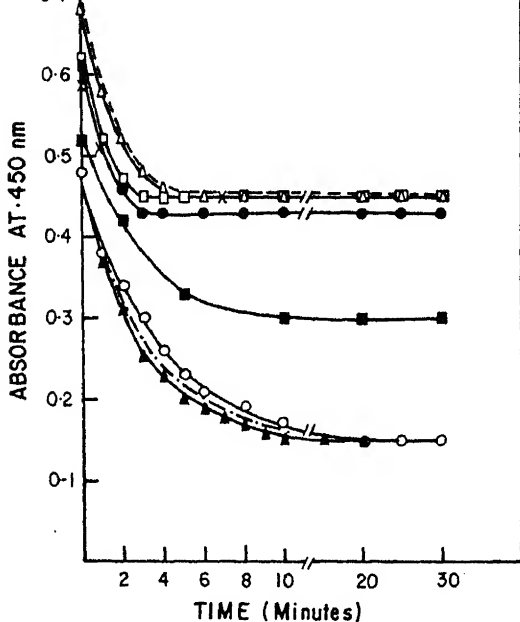


Figure 3. Time course of bacterial cell lysis inhibition by cardiotoxin II ( $0.026 \mu\text{mol/ml}$ ) and its oxidized derivatives ( $0.026 \mu\text{mol/ml}$ ): (O), lysozyme control; ( $\Delta$ ), native cardiotoxin II; ( $\square$ ), cardiotoxin II with 1 Tyr oxidized; ( $\times$ ), cardiotoxin II with 2 Tyr oxidized; ( $\bullet$ ), cardiotoxin II with 3 Tyr oxidized; ( $\blacksquare$ ), cardiotoxin II with 4 Tyr oxidized; ( $\Delta$ ), cardiotoxin II oxidized with performic acid, cardiotoxin II oxidized with performic acid and methionine sulfoxide reverted to methionine; (---), cardiotoxin II oxidized with NCS (see text for details).

spectrophotometric observations at the required wavelengths. The solvent  $0.01 \text{ N H}_2\text{SO}_4$  in which the reagent is stable did not seem to offer any special advantage as reagent consumption to effect the same degree of oxidation as in acetate-formic acid buffer was found by us to be much higher, for lower levels of oxidation, presumably due to low accessibility of tyrosine residues at the lower pH.

At pH 8.5 NBS and NIS give rise to product spectra with peak absorption at 310 nm which is attributable to *o*-dihalogenation of tyrosine residues, but not conversion to 3,5-dihalogenodienone ( $\lambda_{\text{max}}$  260 nm) at the alkaline pH (spectra not shown). At pH 4.0 and 8.5, NCS did not affect the absorption spectrum of cardiotoxin II, whereas NIS at pH 4.0 gave rise to a peak of absorption at 290–300 nm, diminution in the normal phenolate absorption and enhanced absorption at 260 nm and lower wavelengths (spectra not shown). Under acidic conditions, only NBS was found to be best suited for oxidation of tyrosine residues of cardiotoxin II.

The lower molar absorption obtained (7288 and 6780 in formate buffer and  $0.01 \text{ N H}_2\text{SO}_4$ , respectively) for the toxin oxidized with NBS could be due to oxidation of some or all of the tyrosines to intermediates other than the dibromodienone lactone expected from studies on NBS oxidation of much simpler (small) model substrates

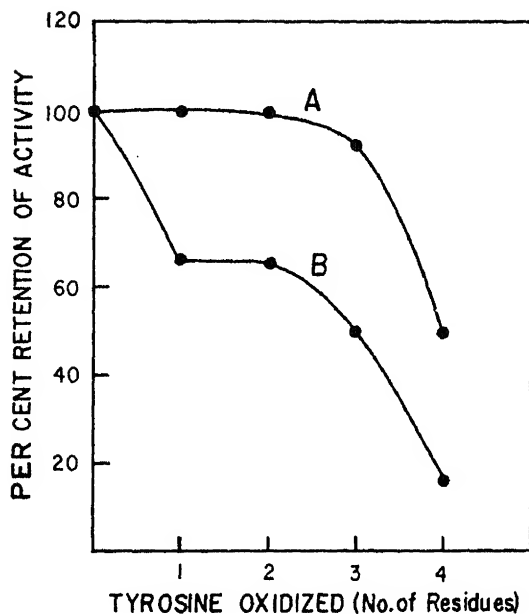


Figure 4. Effect of oxidation of tyrosine residues in cardiotoxin II with NBS on lysis inhibition of *M. lysodeikticus* cells and increase in initial absorbance of bacterial cell suspension. (A), lysis inhibition; (B), increase in absorbance.

(peptides). Even ribonuclease which contains 6 tyrosine residues (Hirs *et al.*, 1960) in its structure, and therefore not ideally suited for comparison with cardiotoxin, cleavage of tyrosyl bonds was achieved by using great excess of oxidant (46 eqs NBS/mol of ribonuclease and 30 eqs/mol) with carboxymethyl-ribonuclease (Wilson and Cohen, 1963). Based on the spectrophotometric data they showed oxidation of only 4.6 tyrosine residues. In the oxidation of cardiotoxin II 2.85 and 2.65 (approx. 3) tyrosine residues appeared to have been oxidized in formate buffer and 0.01 N  $H_2SO_4$ , respectively, based on the extinction expected of dienone ( $E_M$  260–10,200).

Destruction of side chain amino groups of lysozyme by NBS is known (Ramachandran and Witkop, 1959). This loss in side chain amino groups was also noted in cardiotoxin II when treated with excess of NBS (6.25 eqs/tyrosine residue). At concentrations of NBS less than this, which is sufficient to cause oxidation equivalent to that of two tyrosine residues, there was no loss of amino groups.

Cleavage of peptide bonds was ruled out in the present oxidative modification studies based on the absence of formation of any new reactive N-terminal residues by dansylation which offers a sensitive probe. On the contrary, oxidation of performic acid oxidized cardiotoxin with NBS appears to cause some cleavage of peptide bonds. Three extra spots of dansyl amino acids, other than those expected, were seen on the chromatogram relative to the control. O-Dansyltyrosine spots were not detected on the chromatograms of hydrolyzates of oxidized protein indicating that tyrosine residues had undergone modification.

intact -S-S- left untouched by NBS in the modified derivative. However, there was oxidative cleavage of disulphide bonds (table 3) with use of larger amounts of NBS required to oxidize two more of the tyrosine residues present in the toxin.

Carlsson and Louw (1978) had selectively modified methionine residues in cardiotoxin of *Naja melanoleuca* with NCS (8 eqs/mol of toxin) leading to loss in lethal activity. In our studies, toxin treated with 50 eqs of NCS/mol still retained its lethality to mice. Structural differences in the two toxins perhaps account for the difference in susceptibility to loss of biological activity on what is presumed to be oxidation of the two methionine residues in the toxin. On the basis of reactivity (Schechter *et al.*, 1975) to NCS, the only two methionines in cardiotoxin II may be considered as partly, if not fully, buried. Two methionines (24 and 26) are considered invariant in the cardiotoxins (Dufton and Hider, 1983).

It seems reasonable to conclude that the observed loss of lethality of cardiotoxin II, on limited NBS oxidation, results from the oxidation of a single tyrosine residue, whose location in the structure will remain to be ascertained.

The ability of the toxin to confer stability to bacterial protoplasts was not affected when oxidized with lower amounts of NBS. However, a fully oxidized derivative exhibited a retention of only about 50% of such activity. Loss of the remaining 50% of the activity occurs only when the two left-over tyrosine residues, 3 amino groups and two disulphide bonds are oxidatively modified. Marked differences that were observed in the ability of derivatives in bringing about initial increase in absorbance of bacterial cell suspensions may be due to involvement of different structural elements in the toxin in the two functions.

Three-dimensional structural integrity associated with disulphide bonds seems to be important for the protoplast stabilization activity of the toxin. Performic acid oxidized toxin is thus inactive, even though all 10 amino groups remain intact. The basic property of the molecule as such seems inadequate, although with compounds such as spermine and related polyamines, which stabilize protoplasts under hypotonic conditions, this property has been invoked to account for the phenomenon (Tabor, 1962). With fully NBS-oxidized cardiotoxin, which still retains two intact disulphide bonds and 7 of the original 10 amino groups, 50% retention of the ability of the native toxin to protect protoplasts is observed.

Earlier studies using tetranitromethane (Keung *et al.*, 1975; Carlsson, 1980) did not fully establish the importance of tyrosine residues in the biological activity of the cardiotoxins studied, since modified derivatives retained atleast partial biological activity, such as lethal potency, haemolytic activity and cytolytic activity. In contrast, the use of the NBS in oxidation of the tyrosine residues in cardiotoxin of the Indian cobra reveals the importance of a tyrosine residue in relation to lethal activity. The tyrosine residue that is involved could be the equivalent of the invariant residue (Tyr 22) which had been noted in cardiotoxins from species other than *Naja naja* (Joubert, 1976; Ryden *et al.*, 1973). This tyrosine residue, however, seems unimportant for protecting bacterial protoplast membranes against lysis under hypotonic conditions.

## Acknowledgements

Financial assistance from the Indian Institute of Chemical Biology and a Fellowship

- Achyuthan, K. E., Ranganatha Rao, K. and Ramachandran, L. K. (1980) *Indian J. Biochem. Biophys.*, **17**, 228.
- Arakati, M. Z., El-Wahab, M. F. A. and El-Sadr, M. M. (1955) *J. Am. Chem. Soc.*, **77**, 1670.
- Bayer, P. D. (1954) *J. Am. Chem. Soc.*, **76**, 1670.
- Carlsson, F. H. H. (1980) *Biochem. Biophys. Acta*, **624**, 460.
- Carlsson, F. H. H. and Louw, A. I. (1978) *Biochem. Biophys. Acta*, **534**, 322.
- Condrea, E. (1974) *Experientia*, **30**, 121.
- Cuifton, M. J. and Hider, R. C. (1983) *CRC Critical Rev. Biochem.*, **14**, 113.
- Davies, R. (1972) *Methods Enzymol.*, **25B**, 464.
- Cartley, B. S. (1970) *Biochem. J.*, **119**, 805.
- DeWitt, C. H. W., Moore, S. and Stein, W. H. (1960) *J. Biol. Chem.*, **235**, 633.
- Hubert, F. J. (1976) *Eur. J. Biochem.*, **64**, 219.
- Leung, W. M., Leung, W. W. and Kong, Y. C. (1975) *Biochem. Biophys. Res. Commun.*, **66**, 383.
- Patchornik, A., Lawson, W. B., Gross, E. and Witkop, B. (1960) *J. Am. Chem. Soc.*, **82**, 5923.
- Ramachandran, L. K. and Witkop, B. (1959) *J. Am. Chem. Soc.*, **81**, 4028.
- Ramachandran, L. K. and Witkop, B. (1967) *Methods Enzymol.*, **11**, 283.
- Snyder, L., Gabel, D. and Eaker, D. (1973) *Int. J. Peptide Protein Res.*, **5**, 261.
- Schechter, Y., Burstein, Y. and Patchornik, A. (1975) *Biochemistry*, **14**, 4497.
- Shashidharan, P. and Ramachandran, L. K. (1984) *Indian J. Biochem. Biophys.*, **21**, 304.
- Shashidharan, P. and Ramachandran, L. K. (1985) *J. Indian Chem. Soc.*, **62**, 920.
- Shrinivasa, B. R., Achyuthan, K. E. and Ramachandran, L. K. (1982) *Indian J. Biochem. Biophys.*, **19**, 52.
- Wabor, C. W. (1962) *J. Bacteriol.*, **83**, 1101.
- Wilson, T. G. and Cohen, L. A. (1963) *J. Am. Chem. Soc.*, **82**, 564.
- Witkop, B. (1961) *Adv. Protein Chem.*, **16**, 221.



# Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma

S. MAHADEVAN

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

**Abstract.** Lipid thermal transition patterns of the very low density lipoproteins in native and variously treated egg yolk plasma and extracted total very low density lipoproteins lipids have been recorded by differential scanning calorimetry in the temperature range 220–300 K, after lowering the freeze endotherm of free water in the sample with ethylene glycol.

Three distinguishable patterns of lipid endotherms, designated types 1, 2 and 3 were obtained, respectively, from (i) native very low density lipoproteins in egg yolk plasma, (ii) freeze damaged very low density lipoproteins in gelled egg yolk plasma and (iii) extracted total lipids of very low density lipoproteins dispersed in water. Protein-depleted 'lipid core' particles of very low density lipoproteins obtained by exhaustive proteolysis of egg yolk plasma gave type 2 lipid transition pattern suggesting similarities in its lipid association with that of the freeze damaged very low density lipoproteins. Freezing the 'lipid cores' of very low density lipoproteins led to phase separation and gave type 3 lipid transition pattern of water-dispersed, phase-separated total very low density lipoprotein lipids. Relative heat uptake of native very low density lipoproteins in egg yolk plasma was about 15% lower than the freeze damaged sample or of the extracted total lipids.

Treatments which prevented aggregation and gelation of very low density lipoproteins in egg yolk plasma during frozen storage, namely with additives such as glycerol or NaCl, gave subsequent lipid transition pattern intermediate between type 1 and 2, indicating that while very low density lipoprotein aggregation is prevented, additives do not altogether prevent changes in lipid association in these particles.

**Keywords.** Differential scanning calorimetry; very low density lipoproteins; egg yolk plasma lipids.

## Introduction

Hen's egg yolk plasma (EYP), obtained by high speed centrifugation of egg yolk (Schmidt *et al.*, 1956), consists of very low density lipoproteins (VLDL) and lipid-free proteins, the livetins (Cook and Martin, 1969). Irreversible gelation of EYP occurs when stored frozen below  $-6^{\circ}\text{C}$  and thawed, and this has been shown to be due to freeze damage and aggregation of the VLDL component of EYP (Saari *et al.*, 1964; Raju and Mahadevan, 1974). Aggregation of the protein component of VLDL as a consequence of changes in non-covalent lipid-protein association during frozen storage has been suggested as the reason for gelation (Mahadevan *et al.*, 1969; Raju and Mahadevan, 1976). Additives such as glycerol, sugars or NaCl, or treatment with proteolytic enzymes such as trypsin are known to prevent freeze-induced gelation of yolk (Lopez *et al.*, 1955). Their action is believed to be due to the prevention of VLDL protein aggregation either by partial proteolysis or by preventing drastic changes in protein-lipid interaction during frozen storage (Mahadevan *et al.*, 1969).

differential scanning calorimeter. The work was undertaken on the premise that altered physical states of VLDL in EYP and their lipids may manifest themselves with altered patterns of thermal transitions of the lipids, and that a comparison of these patterns may throw light on the nature of lipid-lipid and lipid-protein association in the native and gelled states. The results are discussed in relation to the 'lipid core' model structure proposed for VLDL in EYP based on high resolution nuclear magnetic resonance and other evidences (Kamat *et al.*, 1972; Easwaran and Mahadevan 1972; Easwaran *et al.*, 1980).

## Materials and methods

EYP was prepared from the yolk of fresh white eggs by preparative ultracentrifugation at 137000 *g* for 4.5 h in the cold. The EYP was decanted free of sedimented granules and stored in ice till use.

### *Frozen storage of EYP and induction of gelation*

Native EYP or EYP containing non-gelling additives such as 5% NaCl (w/w), 10% glycerol (w/w) or following trypsin treatment (see below) were stored at  $-25^{\circ}\text{C}$  for a minimum period of 6 days before thawing. While the native EYP had gelled (owing to freeze damage of VLDL in it) the treated samples were still fluid.

For trypsin treatment, about 2 g of EYP was treated with 6.7 mg of trypsin (type III, Sigma Chemical Co., USA) dissolved in 100  $\mu\text{l}$  water and incubated at  $33^{\circ}\text{C}$  for 4 h. Following digestion, 10 mg of soyabean trypsin inhibitor (type 1-S, Sigma Chemical Co., USA) dissolved in 100  $\mu\text{l}$   $\text{H}_2\text{O}$  was added. One half of the trypsinized EYP was stored at  $-25^{\circ}\text{C}$ , while the other half was used immediately in calorimetric studies.

### *Total lipids, phospholipids and neutral lipids*

The total lipids were extracted from lyophilised EYP as described earlier (Easwaran *et al.*, 1980).

For calorimetric studies, one portion of the total lipids was dispersed in enough water to give an opaque yellow emulsion with a lipid concentration equivalent to that in EYP.

A second portion of total lipids was dispersed in excess cold acetone and the precipitate of phospholipids collected by centrifugation in the cold. The precipitate was washed twice with cold acetone and the final pale yellow precipitate dried *in vacuo* and dispersed in a water-glycol mixture (2:1) prior to calorimetry.

A third portion of total lipids, dissolved in redistilled  $\text{CHCl}_3$ , was loaded on a column of silicic acid (25 g of Kieselgel 0.05–0.2 mm; Merck, Germany) in  $\text{CHCl}_3$  in the cold ( $4^{\circ}\text{C}$ ). The column was eluted with cold  $\text{CHCl}_3$  and the initial 150 ml of nearly colourless eluate flash evaporated at  $30^{\circ}\text{C}$ . The pale straw coloured oil residue of neutral lipids, chiefly triglycerides, accounted for over 66% of the total lipids

added. Carotenoids were not eluted with this fraction under these conditions. The neutral lipid fraction was directly used in calorimetric studies.

### *Protease treatment of EYP*

Protein-depleted EYP was prepared by extensive proteolysis of about 8 g of EYP with 53 mg of protease (ex *Streptomyces griseus*) essentially as described by Easwaran *et al.* (1980). After 48 h of proteolysis and dialysis, the material was dialyzed against 0.5 M NaCl solution in the cold for a further 16 h. A sample of EYP with no added protease was treated in exactly the same manner to serve as control.

A part of the protease-treated EYP and control EYP were analyzed for protein content. Calorimetric studies were conducted on the protease-treated EYP prior to and following frozen storage at  $-25^{\circ}\text{C}$  for 48 h.

### *Protein estimation*

Lipid-rich native and protease-treated EYP samples were analyzed for proteins by a slight modification of the Folin's procedure as described by Easwaran *et al.* (1980). Optical density was measured at 500 nm in a Unicam SP 600 spectrophotometer, using crystalline bovine serum albumin as the standard.

### *Differential scanning calorimetry (d.s.c.)*

Differential thermal transition heating curves were obtained with a Perkin-Elmer differential scanning calorimeter DSC-2 using helium as the purge gas in the 'head' and nitrogen in the outside chamber. Liquid nitrogen was used as the sample coolant. In order to obtain comparable results, all measurements were made with the following settings: cooling rate:  $320^{\circ}/\text{min}$ ; heating rate:  $5^{\circ}/\text{min}$  and 1 m cal/s full scale sensitivity. All measurements were recorded up to 300 K ( $27^{\circ}\text{C}$ ) since initial experiments indicated no thermal transitions beyond this temperature except that of thermal denaturation of EYP.

Samples (10–15 mg) containing either 23 or 33% ethylene glycol were sealed in aluminium pans. Gelled EYP samples mixed with glycol gave a foamy paste which was clarified by centrifugation at 40000 g for 10 min. Samples were cooled to varying temperatures (220–270 K) prior to heating and were always maintained at the lower temperature for at least 5 min before heating.

Relative heat uptake was calculated from the area under the d.s.c. curves obtained with a planimeter.

## **Results and discussion**

Initial experiments indicated that fresh EYP gave no thermal transitions when heated from its freezing point (272 K) to temperature of thermal denaturation. However on freezing to 220 K before heating, an endothermic transition peak at

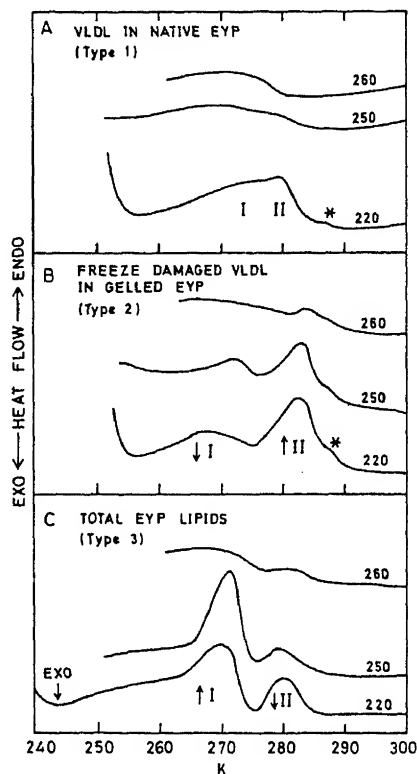


protein components.

Lipid transitions occurring at the water melting temperature were thereafter obtained by the addition of ethylene glycol. This additive was useful as it also suppressed freeze induced gelation of EYP. Preliminary trials indicated that most samples could undergo a few cycles of freeze-thaw in the d.s.c. while registering identical thermal transition pattern. Fresh samples were used whenever such cycles started inducing a change in the endotherm pattern.

### *Thermal transition pattern (type 1) of native VLDL in EYP*

Figure 1A shows transition curves obtained from native VLDL in fresh EYP containing ethylene glycol (23% final concentration) cooled to 220, 250 or 260 K before



**Figure 1.** A. D.s.c. heating curves (type 1) of native VLDL in EYP sample containing ethylene glycol (23% final conc.). Sample temperature before heating indicated on each curve. I and II represent the two regions of the curve described in text. Abscissa-K (degrees absolute). Ordinate-heat flow; upward endothermic and downward exothermic. B. Type 2 transitions of freeze damaged VLDL in gelled EYP sample obtained by frozen storage of EYP at  $-25^{\circ}\text{C}$  for 11 days before thawing. Sample contained 23% ethylene glycol. Arrows represent relative shifts of I and II regions compared to A. C. Type 3 transitions of total EYP lipids dispersed in water and containing 33% glycol. Arrows represent relative shift of I and II compared to B. Exo. Exothermic transition

ating. At 250 K and above broad nondescript transitions were obtained. At 220 K, transition starting around 254 K and levelling off as a shoulder (I) at 274 K followed by a small peak (II) at 280 K was obtained with a small shoulder at 288 K (asterisk). This pattern of thermal transition is designated as type 1. Use of 33% glycol instead of 23% glycol gave a similar pattern except that the onset of transition at 252–253 K was more clearly seen.

#### *Thermal transition pattern (type 2) of freeze damaged VLDL in gelled EYP*

The thermal transition pattern of freeze damaged VLDL in gelled EYP (figure 1B) was clearly different from that of native VLDL in EYP and is designated as type 2. With the sample frozen to 220 K, a two peaked pattern was obtained, a first small peak (I) at 267 K and a second larger peak (II) at 282 K. A small shoulder (asterisk) at 288 K was also seen. In comparison to type 1, type 2 showed separation of peaks I and II and a relative reduction in height of I compared to II (arrows in figure 1B). Unlike type 1, type 2 gave similar transition, though of reduced magnitude even when cooled to 250 K. However the pattern broadened when cooled to 260 K as in type 1.

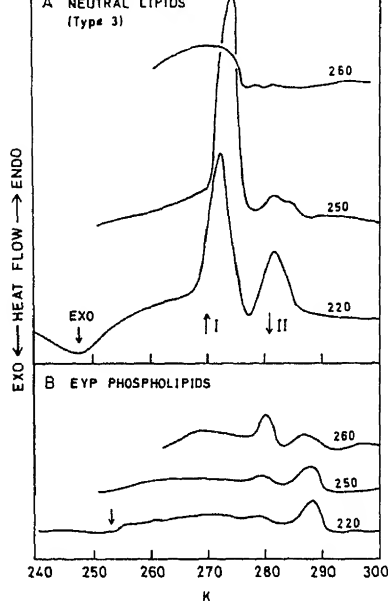
#### *Thermal transition pattern (type 3) of total EYP lipids*

The thermal transition patterns for total extracted EYP lipids dispersed in water and glycol (figure 1C) were qualitatively different from types 1 and 2 and is termed as type 3. Cooled to 220 K, type 3 pattern showed a large gradually rising curve peaking at 270 K (I) followed by a smaller peak II at 281 K. The peaks were closer and relative heights reversed compared to type 2 (arrows in figure 1C). An exothermic transition at 243 K was also seen. With sample cooled to 250 K, peak I shifted to 272 K and became sharper and larger while peak II diminished. Cooling to 260 K gave broad transitions as in types 1 and 2.

These patterns were qualitatively similar to transitions obtained with neat, neutral fraction (chiefly triglycerides) of EYP lipids as given in figure 2A. With sample cooled to 220 K, an exothermic transition at 243 K, a slowly rising large endothermic transition peaking at 273 K (I) and a smaller 282 K peak (II) were obtained. Cooling to 250 K showed similar increase of I and lowering of II as in type 3, while cooling to 260 K gave the broadened pattern. The dependence of the pattern of endothermic transitions on the degree of cooling is undoubtedly due to the various polymorphic forms that these mixtures of triglycerides can crystallize into (Ladbrooke and Chapman, 1964).

The phospholipids fraction (figure 2B) in water and glycol, when cooled to 220 K or 250 K) gave a very broad transition from 253 K ending with a minor peak at 288 K. When starting temperature was increased to 260 K, the peak at 288 K decreased and a new one at 280 K was obtained. This transition at 288 K is apparently visualized as a minor shoulder in native and freeze damaged (types 1 and 2) samples in figure 1 (asterisk).

#### *Thermal transition pattern of protease treated 'lipid core' of VLDL*



**Figure 2.** A. D.s.c. heating curves of neutral EYP lipids (triglycerides) in the neat form. B. Transitions of phospholipid fraction of EYP dispersed in water and containing 33% glycol. Other details as in figure 1.

obtained by exhaustive proteolysis of EYP with concomitant dialysis. Protein analysis before or after proteolysis indicated that about 2–3% of the original proteins remained (Easwaran *et al.*, 1980). The thermal transition patterns of 'lipid core' of VLDL are given in figure 3A. With samples cooled to 220 K, a small peak I at 268 K and a larger peak II at 272 K resembling type 2 transition of freeze damaged VLDL in EYP (figure 1B) was obtained. Cooling to 250 K retained the pattern while at 260 K the pattern broadened but still discernible when compared to the freeze damaged VLDL sample. Removal of proteins of VLDL therefore yielded 'lipid core' particles where lipids were associated in a manner similar to lipids in freeze damaged VLDL in gelled EYP (type 2) and unlike that in native VLDL (type 1).

Protein depleted 'lipid core' particles stored frozen for 2 days at  $-25^{\circ}\text{C}$  and thawed changed from a translucent yellow fluid to an opaque yellow; viscous material resembling solvent extracted EYP lipids dispersed in water. The d.s.c. curves of frozen and thawed 'lipid core' cooled to 220 K (figure 3B) resembled type 3 pattern of total EYP lipids with peak I larger than peak II and this difference becoming greater when cooled to 250 K prior to heating. Freeze damage (by phase separation) of 'lipid core' of VLDL occurred even in the presence of 33% of glycol and gave similar transition patterns.

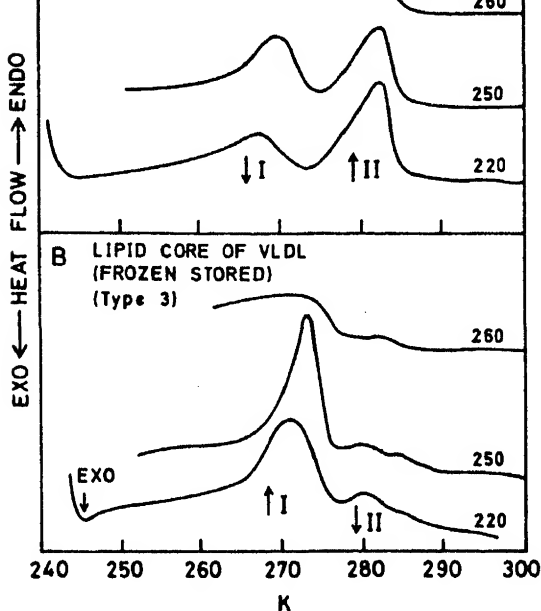


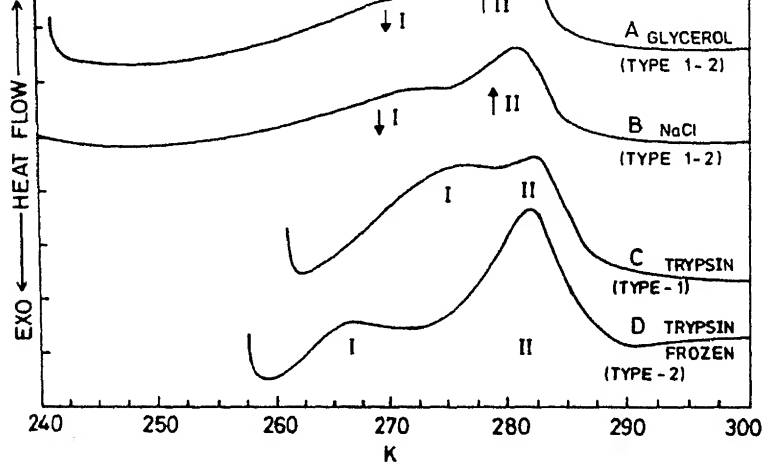
Figure 3. A. D.s.c. heating curves of a protease-treated 'lipid-core' of EYP containing 33% glycol. B. Transition curves of protease-treated 'lipid-core' of EYP stored frozen at  $-25^{\circ}\text{C}$  for 2 days before addition of glycol (33% final conc.). Other details as in figure 1.

between type 1 and type 2, with peak II larger than I as in type 2, but I appearing as a shoulder of II as in type 1. Obviously these additives while preventing gelation did not altogether prevent changes in lipid association.

Partial proteolysis of EYP by trypsin treatment prior to frozen storage also prevented gelation. In figure 4, transition curves of trypsinized EYP samples before frozen storage (curve C) and after frozen storage (curve D) are shown, the former identical to native EYP (type 1) while the latter similar to the freeze damaged VLDL in gelled EYP (type 2). Apparently partial degradation of VLDL proteins prevented gel formation but not changes in lipid association during frozen storage.

*Relative heat uptake by VLDL in variously treated EYP and of lipids extracted from EYP*

Table 1 gives the per cent heat uptake by VLDL in and variously treated EYP samples and of extracted lipids relative to native VLDL in fresh EYP. All values were calculated from the area under the curve for samples cooled to 220 K prior to heating. The enthalpy of lipid transition of native VLDL in fresh EYP was about 10% of that observed for VLDL in frozen stored EYP samples, whether gelled, or un-gelled by additives, or for the total extracted lipids. While the freeze damaged 'lipid core' had similar enthalpy as other frozen stored samples, unfrozen, protein depleted 'lipid core' of VLDL showed a relative heat uptake intermediate between native VLDL in fresh EYP and the other frozen samples.



**Figure 4.** D.s.c. heating curves at EYP samples treated with various non-gelling agents prior to frozen storage. All samples cooled to 220 K before heating. EYP samples containing either 10% glycerol (w/w) (A) or 5% NaCl (w/w) (B) before thawing, and stored for 7 days at  $-25^{\circ}\text{C}$ . Glycol (33% final conc.) added prior to d.s.c. run. Curves C and D-EYP samples treated with trypsin and trypsin inhibitor as described in 'materials and methods'. C-unfrozen sample with 23% glycol; D-sample frozen for 6 days at  $-25^{\circ}\text{C}$  before addition of 23% glycol.

**Table 1.** Relative heat uptake during lipid thermal transition by native and variously treated EYP and EYP lipids.

Sample	Per cent
Native VLDL in fresh EYP	100
Freeze damaged VLDL in frozen stored (gelled) EYP	118
Total lipids of EYP dispersed in water*	117
Phospholipids of EYP dispersed in water*	23
'Lipid core' of VLDL (protease treated EYP)*	108
'Lipid core' of VLDL (frozen stored-phase separated)*	120
EYP (frozen stored) in 10% glycerol	117
EYP (frozen stored) in 5% NaCl	118

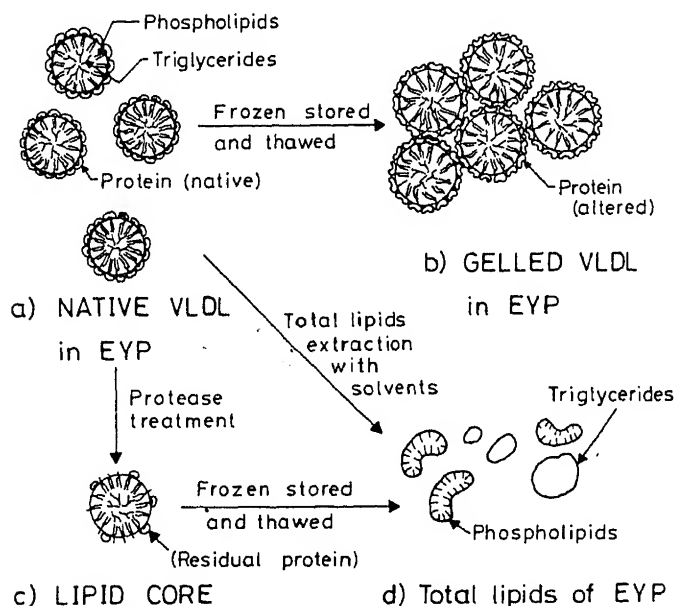
\*Calculated on equivalent EYP basis.

A comparison of the thermal transition patterns of native (type 1) or freeze damaged (gelled) VLDL in EYP (type 2) and of total lipids extracted from EYP (type 3) in the temperature range described indicate that the endotherms are primarily due to the lipids, particularly the abundant neutral triglycerides. However, the differences in the two major zones of transitions I and II, clearly indicate that the lipids can exhibit at least 3 different polymorphic forms which are undoubtedly dependent on differences in lipid-lipid and possibly lipid-protein association states.

Of these 3, type 1 lipid association is unique in having a lower enthalpy of transition probably due to protein lipid interactions in the native VLDL particles. Steim *et al.* (1969) have reported a 25% lower enthalpy of transition of the lipids in the membranes of *Achyloplasma laidlawii* as compared to lipids extracted from it, and the difference has been attributed to the interaction of proteins with a fraction of the phospholipids of the membrane. High resolution NMR studies have suggested protein interactions with lecithin head group in VLDL particles of EYP (Easwaran *et al.*, 1980). A lesser interaction with surface proteins occurs in type 2 since protein-depleted 'lipid cores' of VLDL gave a pattern similar to the freeze damaged VLDL in EYP. Type 3 lipid association in phase separated, extracted total lipids has no interactions with proteins at all, and a similar pattern is obtained from frozen stored, phase separated 'lipid cores' of VLDL.

Based on these results, the gross structure for native, freeze damaged (gelled) and protein depleted 'lipid cores' of VLDL in EYP are shown schematically in figure 5. Native VLDL in fresh EYP (figure 5a) is represented (in cross section) as discrete large spheres with a core of triglycerides in the centre and monolayer of phospholipids at the surface oriented radially. The polar head groups of the phospholipids at the surface of the sphere is in close association with the VLDL proteins around the lipid core as inferred from proton magnetic resonance studies (Easwaran *et al.*, 1980).

The similarity of transition patterns of protein-depleted 'lipid-core' particles of VLDL and freeze damaged (gelled) VLDL (*i.e.* type 2), suggests that lipid association



action in the former, it is suggested that negligible protein-lipid interaction occurs in the latter also. Freeze damaged VLDL particles are therefore represented (figure 5c) with a lipid core similar to protein-depleted 'lipid core' particles having surface proteins with an altered conformation which reduces their interaction with the lipids but increases intra or inter-particle protein interaction leading to aggregation and eventual gelling. Protein fractions with an ability to form gels even in low concentrations have been isolated from VLDL (Raju and Mahadevan, 1975).

Though the protein-lipid interaction in the gelled particles was less than in native VLDL particles, there were still enough influence of the protein to prevent phase separation in the lipids to give type 3 transitions, which both extracted lipids and frozen protease-treated 'lipid cores' of VLDL gave. The proteins therefore prevented large scale aggregation of the discrete lipid core units needed for a phase separation.

Both total EYP lipids in water and frozen stored protease treated 'lipid core' of VLDL are depicted as a conglomeration of various sized particles following phase separation of the neutral and phospholipids (figure 5d), the former existing as globules of fat and the latter as bilayered vesicles or other ordered forms resulting in some crystallinity. A preliminary low angle X-ray scattering experiment suggested some crystallinity in the phase separated sample (unpublished observations). Owing to the large variety of lipid molecules with varying fatty acid composition, the ordered structures would themselves be a mosaic of patterns.

Gelation of VLDL of EYP following freeze damage therefore appeared to be chiefly due to protein aggregation following disruption or alteration in lipid-protein interaction. This leads to alterations in lipid-lipid interaction but these changes are not sufficient to cause drastic differences in viscosity since frozen stored, trypsin-treated EYP was still fluid, though the lipid thermal transition pattern was similar to that of gelled EYP. Additives like NaCl and glycerol while preventing gelation of EYP on frozen storage, did not altogether prevent changes in lipid association.

## Acknowledgement

Much of this work was done in the laboratory of Prof. D. Chapman, Department of Chemistry, University of Sheffield, Sheffield, UK during 1973 when the author was supported by a visitorship of the British Council, UK and a travel grant from the University Grants Commission, New Delhi.

The author thanks Prof. Chapman and Dr. V. B. Kamat for stimulating discussions and Dr. Bryan Cater and Mrs. Sue Hawes for initial help with the d.s.c.

Research partly financed by a grant from the US Department of Agriculture, under PL-480 Project No. FG-In-477.

## References

- Cook, W. H. and Martin, W. G. (1969) in *Structure and functional aspects of lipoproteins in living systems* (eds E. Tria and A. M. Scanu) (London: Academic Press) p. 579.
- Easwaran, K. R. K. and Mahadevan, S. (1972) *FEBS Lett.*, **25**, 134.
- Easwaran, K. R. K., Raju, K. S. and Mahadevan, S. (1980) *J. Biosci.*, **2**, 1.
- Kamat, V. S., Lawrence, G. A., Barratt, M. D., Darke, A., Leslie, R. B., Shipley, G. G. and Stubbs, J. M.

- Ladbrooke, B. D. and Chapman, D. (1969) *Chem. Phys. Lipids*, **3**, 304.
- Lopez, A., Fellers, C. R. and Powrie, W. D. (1955) *J. Milk Food Technol.*, **18**, 77.
- Mahadevan, S., Satyanarayana, T. and Kumar, S. A. (1969) *J. Agric. Food. Chem.*, **17**, 767.
- Raju, K. S. and Mahadevan, S. (1974) *Anal. Biochem.*, **61**, 538.
- Raju, K. S. and Mahadevan, S. (1976) *Biochim. Biophys. Acta*, **446**, 387.
- Saari, A., Powrie, W. D. and Fennema, O. (1964) *J. Food. Sci.*, **29**, 762.
- Schmidt, G., Bessman, H. J., Hickey, M. D. and Thannhauser, S. J. (1956) *J. Biol. Chem.*, **223**, 1027.
- Steim, J. M., Tourtellotte, M. E., Reinert, J. C., McElhanney, R. N. and Rader, R. L. (1969) *Proc. Natl. Acad. Sci. USA*, **63**, 104.





## Amphomycin: A tool to study protein N-glycosylation

DIPAK KUMAR BANERJEE

Department of Biochemistry and Nutrition, School of Medicine, University of Puerto Rico, San Juan, Puerto Rico 00936-5067, USA

**Abstract.** Radio-labelled amphomycin ( $^3\text{H}$ -amphomycin) forms a complex with dolichylmonophosphate in presence of  $\text{Ca}^{2+}$ . Complex formation has also been documented with retinylmonophosphate and perhydromonoeneretinylnonophosphate. Analysis of the space-filling model suggested both fatty acylated aspartic acid residue at the N-terminus of the lipopeptide and phosphate head group of dolichylmonophosphate are necessary for the complex formation. The binding ability of amphomycin is then utilized to localize dolichylmonophosphate in the microsomal membrane. Studies with microsomal membranes from hen oviduct suggested that dolichylmonophosphate is located in the cytoplasmic side of the membrane.

**Keywords.** Glycosylation inhibitor; protein N-glycosylation; dolichylmonophosphate; lipid-linked pathway.

### Introduction

Many of the glycoproteins from eukaryotic cells contain oligosaccharide chains that are attached to protein *via* GlcNAc→asparagine linkage (Kornfeld and Kornfeld, 1985). Detail structural analysis of these oligosaccharides indicated that they fall into 3 major categories termed high mannose, hybrid and complex. They all share the common core structure Man  $\alpha$ 1-3 (Man  $\alpha$ 1-6) Man  $\beta$ 1-4 GlcNAc  $\beta$ 1-4 GlcNAc-Asn, but differ in outer branches. The high mannose-type oligosaccharides typically have 2 or 6 additional mannose residues linked to the pentasaccharide core. The hybrid molecules have features of both high-mannose and complex-type oligosaccharides. Most hybrid molecules contain a 'bisecting' N-acetylglucosamine linked  $\beta$ 1,4 to the  $\beta$ -linked mannose residue, although there are some exceptions (Hunt *et al.*, 1983; Varki and Kornfeld, 1983; Yamashita *et al.*, 1983). The complex-type structure contains two outer branches with the typical sialyl lactosamine sequence and shows two other commonly found substituents, namely a fucose in  $\alpha$ 1,6 linkage to the innermost N-acetylglucosamine residues and a 'bisecting' N-acetylglucosamine linked  $\beta$ 1,4 to the  $\beta$ -linked mannose residue.

All types of asparagine-linked carbohydrate chains are initially synthesized as precursor oligosaccharides containing glucose, mannose and N,N'-diacetylchitobiose on a pyrophosphorylated lipid backbone called dolichol. Many aspects of this lipid-mediated process have been reviewed (Lucas and Waechter, 1976; Waechter and Lennarz, 1976; Hemming, 1977; Parodi and Leloir, 1979) but all the details in the synthesis of these oligosaccharides are still not known. One approach to examining the

GlcNAc-1-P to dolichylmonophosphate (Dol-P) (Tkacz and Lampen, 1975; Takatsuki *et al.*, 1975; Struck and Lennarz, 1977; Waechter and Harford, 1977; Ericson *et al.*, 1977), the biosynthesis of the dolichol-linked oligosaccharides is blocked completely. Thus, tunicamycin becomes an useful tool for studying the biosynthesis, metabolic fate, and activity of the unglycosylated forms of glycoproteins normally containing N-glycosidically bound oligosaccharides. However, this antibiotic apparently has no effect on the synthesis of mannosylphosphoryl dolichol (Man-P-Dol) or glucosylphosphoryl dolichol (Glc-P-Dol) and therefore has limited warranty for studying intermediate stages in the assembly of dolichol-linked oligosaccharides.

The antibiotic amphomycin produced by *Streptomyces canas* (Hainemann *et al.*, 1953) is a straight chain undecapeptide with either 3-isododecenoic or 3-anteisotridecenoic acid residue attached to the N-terminal aspartic acid residue by an amide linkage (Bodanszky *et al.*, 1973). Earlier it has been shown by Tanaka *et al.* (1977, 1979) that amphomycin inhibits the synthesis of peptidoglycan in gram-positive bacteria by blocking the transfer of phospho-N-acetyl-muramylpentapeptide from UMP to undecaprenylmonophosphate, the prokaryotic glycosyl carrier lipid. Considering the structural relationship between undecaprenylmonophosphate and Dol-P, it is reasonable to expect that the antibiotic would also affect glycosylation of the eukaryotic carrier lipid. Studies with isolated microsomal membrane preparations from pig aorta and plants have, indeed, demonstrated that glycosylation reactions involving Dol-P are inhibited by amphomycin (Kang *et al.*, 1978a,b; Ericson *et al.*, 1978; Kang and Elbein, 1979). Later on Banerjee *et al.* (1981) have demonstrated that inhibition of Man-P-Dol, Glc-P-Dol and N-acetylglucosaminylpyrophosphoryl dolichol (GlcNAc-PP-Dol) synthesis in calf brain microsomal membranes is reversed by the addition of exogenous Dol-P but not by sugar nucleotides or divalent cations. Furthermore, amphomycin also shown to interfere with the extraction of exogenous prelabelled Dol-<sup>32</sup>P but not [<sup>3</sup>H]-Man-P-Dol or the major membrane phospholipids indicating, thereby an interaction between the lipopeptide and dolichylmonophosphate.

This paper presents evidence that amphomycin inhibits monosaccharide-lipid synthesis primarily due to its direct interaction with the glycosyl carrier lipid, Dol-P.

## Materials and methods

Dolichylmonophosphate was purchased from Calbiochem. Dolichol, Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Bio-Gel A-1.5 m (200–400 mesh), Bio-Gel P-2 (200–400 mesh) were obtained from Bio-Rad. [<sup>3</sup>H]-KBH<sub>4</sub> (1.1 Ci/mmol) was supplied by ICN Pharmaceuticals Inc, Irvine, California, USA. Amphomycin (calcium salt) was a gift from Bristol Laboratories and Dr. M. Bodanszky, Case Western Reserve University. Retinylmonophosphate and perhydromonoeneretiny-monophosphate were gift from Dr. L. M. DeLuca, National Cancer Institute. All other chemicals and reagents were analytical reagent grade. For all purposes amphomycin was dissolved in 0.1 N acetic acid, and the solution was adjusted to 0.05 M sodium acetate, pH 7.0 with 0.2 N NaOH.

### Tritium labelling of amphomycin

Isotopic labelling of amphomycin was carried out at the free-NH<sub>2</sub> group of  $\alpha$ -aminobutyric acid residue. The method involves reductive methylation using formaldehyde and high specific activity [<sup>3</sup>H]-potassium borohydride, essentially the same as described before (Kumarasamy and Symons, 1979) for proteins. In most cases 100  $\mu$ g of amphomycin was taken in 500  $\mu$ l of 0.2 M sodium borate, pH 9.5, cooled in ice and to this ice-cold solution was added 3  $\mu$ l of 20 mM formaldehyde, followed after 30 s by 1  $\mu$ l (160  $\mu$ Ci) of [<sup>3</sup>H]-KBH<sub>4</sub> in 10 mM KOH. After 20 min, 1  $\mu$ l of unlabelled 10 mM NaBH<sub>4</sub> was added and the reaction mixture left for a further 20 min at 0°C. The reaction was stopped by adding 200  $\mu$ l of 0.4 M sodium phosphate, pH 5.8 and 100 mM glycine. The pH of the solution was then adjusted to 7.0 and amphomycin was extracted with n-butanol. The organic phase was washed with water and dried under N<sub>2</sub>. Finally, the labelled lipopeptide antibiotic (26.7 mCi/mmol) was dissolved in 0.05 M sodium acetate, pH 7.0 and stored at -20°C until use.

### SG-81 Paper chromatography of [<sup>3</sup>H]-amphomycin

Tritium-labelled as well as unlabelled amphomycin was chromatographed on EDTA-treated SG-81 paper (Steiner and Lester, 1972) and developed with (i) n-butanol-acetic acid-water (3:1:1); (ii) n-propanol-water (7:3) (Bodanszky *et al.*, 1973). Radioactivity was detected by spraying the paper with enhance spray (New England Nuclear, Boston, Massachusetts, USA) followed by exposing XAR-5 film at -80°C (Bonner and Laskey, 1974) and or cutting 1 cm area and counting them in a liquid scintillation counter. For normal visualization, the paper was sprayed with 3% ninhydrin in acetone.

### Measurement of radioactivity

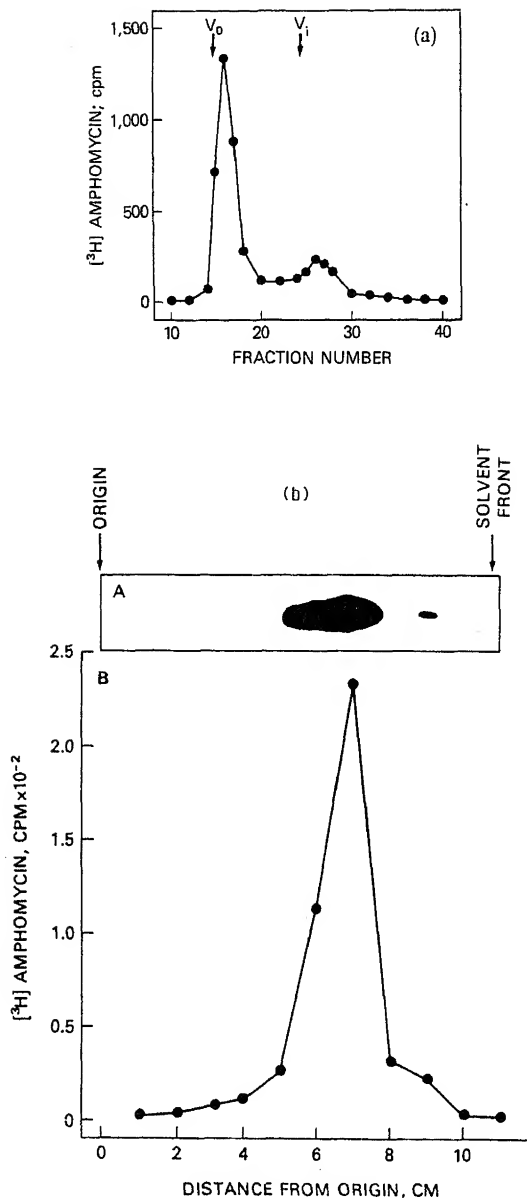
All radioactive samples were counted in Hydrofluor (National Diagnostics).

## Results

### Purity of [<sup>3</sup>H]-amphomycin

The purity of the labelled antibiotic was established by column chromatography on Bio-Gel P-2 as well as by SG-81 paper chromatography. As shown in figure 1a [<sup>3</sup>H]-amphomycin is included in P-2 column (23  $\times$  0.64 cm) and eluted out as single peak. Figure 1b shows that [<sup>3</sup>H]-amphomycin migrates as single species with an R<sub>f</sub> of 0.64. This migration pattern is identical to that observed before (Bodanszky *et al.*, 1973). These criteria established the fact that the radiolabelled amphomycin is extremely pure.

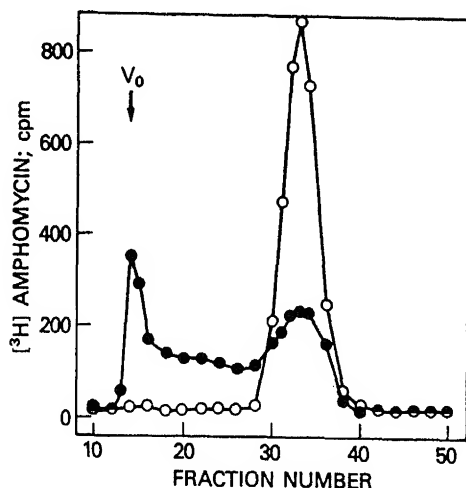
### Interaction of [<sup>3</sup>H]-amphomycin with delipidylmonophosphate



**Figure 1.** (a), Bio-Gel P-2 column chromatography of  $[^3\text{H}]$ -amphomycin. Amphomycin was labelled by reductive methylation and 7,500 cpm were applied to a Bio-Gel P-2 column (23 cm  $\times$  0.64 cm). The column was equilibrated and washed with 0.2 M NaCl. 0.5 ml fractions were collected and counted. ( $V_0$ ), Blue dextran, ( $V_i$ ),  $[^{14}\text{C}]$ -leucine.

(b), SG-81 paper chromatography of  $[^3\text{H}]$ -amphomycin. 10,000 cpm of  $[^3\text{H}]$ -amphomycin were spotted on SG-81 paper. The chromatogram was developed in n-butanol-acetic acid-water (3:1:1). The paper was then sprayed lightly with enhance spray and exposed XAR-5 film for two weeks (upper) at  $-80^\circ\text{C}$ . The radioactivity was also followed by cutting 1 cm area from the original chromatogram and counting them in a liquid scintillation counter.

temperature with 50  $\mu\text{g}$  of Dol-P in presence of 10 mM  $\text{CaCl}_2$  and 0.05% Triton X-100. The reaction mixture was then analyzed on a Bio-Gel A-1.5 m column. The elution profile in figure 2 indicated that a major portion of the radioactivity was eluted in the void volume which suggested the formation of a soluble complex between the lipopeptide and the lipid.

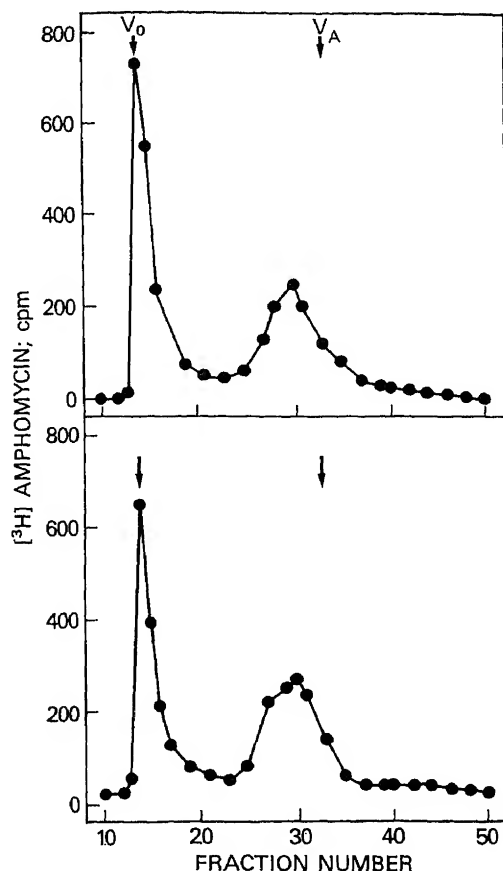


**Figure 2.** Bio-Gel A-1.5 m chromatography of [<sup>3</sup>H]-amphomycin and Dol-P complex. 50  $\mu\text{g}$  of Dol-P was suspended by sonication in 20 mM Tris-HCl buffer, pH 7.0 containing 0.05% Triton X-100. It was then mixed with 5,000 cpm of [<sup>3</sup>H]-amphomycin in the presence of 10 mM  $\text{CaCl}_2$ . The mixture was then applied to a Bio-Gel A-1.5 m column (25 cm  $\times$  0.64 cm). The column was equilibrated and washed with 20 mM Tris-HCl, pH 7.0 containing 10 mM  $\text{CaCl}_2$ , 0.05% Triton X-100. 0.5 ml fractions were collected and counted.  $V_0$  = Blue dextran. (○), [<sup>3</sup>H]-amphomycin; (●), [<sup>3</sup>H]-amphomycin complexed with Dol-P.

### Complex formation of [<sup>3</sup>H]-amphomycin with retinylphosphate and perhydromonoeneretinyphosphate

The possible connection between Dol-P and retinylmonophosphate (RP) systems are due to the facts that both Dol-P and chemically synthesized all-trans- $\beta$ -retinylphosphate; all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-tetraphenyl-1-yl phosphate; perhydromonoeneretinyphosphate; all-trans- $\beta$ -retinylphosphate and 13-cis- $\beta$ -retinylphosphate were acted as acceptors of [<sup>14</sup>C]-mannose from GDP [<sup>14</sup>C]-mannose (De Luca *et al.*, 1977). But when the mannosyldonor activity of both mannosylphosphorylretinol (MRP) and mannosylphosphorylperhydromonoeneretinyphosphate (pMRP) were studied, it was found that mannose could be transferred only from MRP to protein but not from pMRP (M. De Luca, unpublished results). Recently, it had been observed that mannosyl transfer reaction from GDP-mannose to retinylphosphate with rat liver microsomal

analyzed on a Biorad G50 column. The results from such experiments are depicted in Figure 3.

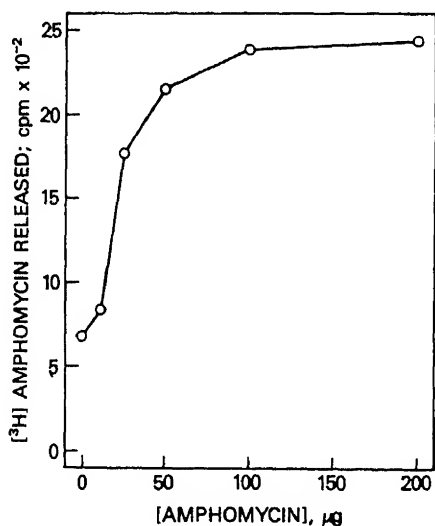


**Figure 3.** Interaction between [<sup>3</sup>H]-amphomycin and retinylphosphate/perneretinyphosphate. 94  $\mu$ g of retinylphosphate (upper panel) and 94  $\mu$ g of perneretinyphosphate (lower panel) were suspended by sonication in equilibrating buffer mixed with 4,000 cpm of [<sup>3</sup>H]-amphomycin at room temperature. The mixture was applied to a Bio-Gel A-1.5 m column (25 cm  $\times$  0.64 cm) which was washed with Tris-HCl, pH 7.0 containing 10 mM  $\text{CaCl}_2$ , 0.05% Triton X-100. 0.5 ml fractions were collected and counted. ( $V_0$ ), Blue dextran; ( $V_A$ ), [<sup>3</sup>H]-amphomycin.

### *Localization of Dol-P in the microsomal membranes*

In the recent past, a few attempts had been made either by protease treatment of liver microsomes and following the synthesis of Man-P-Dol, Glc-P-Dol, GlcNAc-P-Dol as well as addition of glucose to oligosaccharide-lipid (Snider *et al.*, 1981) or by incorporating GlcNAc-PP-Dol into unilamellar liposomes (Hanover and

78) to understand the transmembrane location of oligosaccharide-lipid synthesis and also the topological orientation of N,N'-diacetylchitobiosylpyrophosphoryl-malchol in the membrane. But the localization and orientation of the glycosyl carrier-Dol-P in these membranes were not elucidated. Following the direct interaction between amphomycin and Dol-P, a competitive binding of [ $^3\text{H}$ ]-amphomycin with hen oviduct microsomal membranes was performed. In this case membranes were pre-incubated for 5 min at 37°C in presence of 0–200  $\mu\text{g}$  unlabelled amphomycin followed by [ $^3\text{H}$ ]-amphomycin (5,000 cpm) for another 5 min. At the end of the second incubation, the supernatant was analyzed for radioactivity. Only 5% of the total radioactivity was recovered from the membranes pre-incubated in presence of unlabelled amphomycin. The gradual increase in radioactivity, however, was detected in the supernatant when membranes were incubated with increasing amount of amphomycin. Approximately, 50% of the total radioactivity was recovered from the membrane preparation when pre-incubated with 200  $\mu\text{g}$  amphomycin (figure 4).



**Figure 4.** Competition between [ $^3\text{H}$ ]-amphomycin and unlabelled amphomycin for binding to microsomal membranes from hen oviduct. 22.5 mg membrane protein was incubated in 50 mM Tris-HCl, pH 7.5 buffer containing 10 mM  $\text{CaCl}_2$  and 0–200  $\mu\text{g}$  unlabelled amphomycin at 37°C for 5 min in a total volume of 100  $\mu\text{l}$ . At the end of pre-incubation period 5,000 cpm of [ $^3\text{H}$ ]-amphomycin was added in each tube and reincubated for additional 5 min. At the end of the incubation period the tubes were centrifuged at 39,000  $g$  for 10 min and the radioactivity was measured in the supernatant.

## Discussion

We have shown earlier that amphomycin inhibited Man-P-Dol, Glc-P-Dol and GlcNAc-PP-Dol synthesis in calf brain membranes in a dose dependent manner



the interaction between the lipopeptide and polyisoprenylmonophosphate. As a consequence of this interaction, Dol-P in the microsomal membrane has also been investigated.

Tritium labelling of amphomyein proved to be an important tool in studying the lipid-lipopeptide interaction. SG 81 paper chromatography as well as Bio-Gel P-2 column chromatography demonstrated the purity of the labelled product. No difference in migration rate between the unlabelled amphomyein ( $R_f$  0.64) and [ $^3\text{H}$ ]-amphomyein has been observed in n-butanol-acetic acid-water (3:1:1) system. However, it generated a faster moving product ( $R_f$  0.73) when treated with 0.25 N acetic acid at 100 °C for 3 h, a condition that has been shown to liberate the fatty acylated aspartic acid residue at the N-terminus (Bodanszky *et al.*, 1973).

When [ $^3\text{H}$ ]-amphomyein was allowed to interact with Dol-P and monitored over Bio-Gel A-1.5 m column, it has been observed that amphomyein forms a soluble complex with Dol-P. It also forms a complex with retinylphosphate as well as with perhydromonoeneretiny phosphate. The lipid-lipopeptide interaction is found to be dependent upon the presence of  $\text{Ca}^{2+}$  in the reaction mixture, because inclusion of

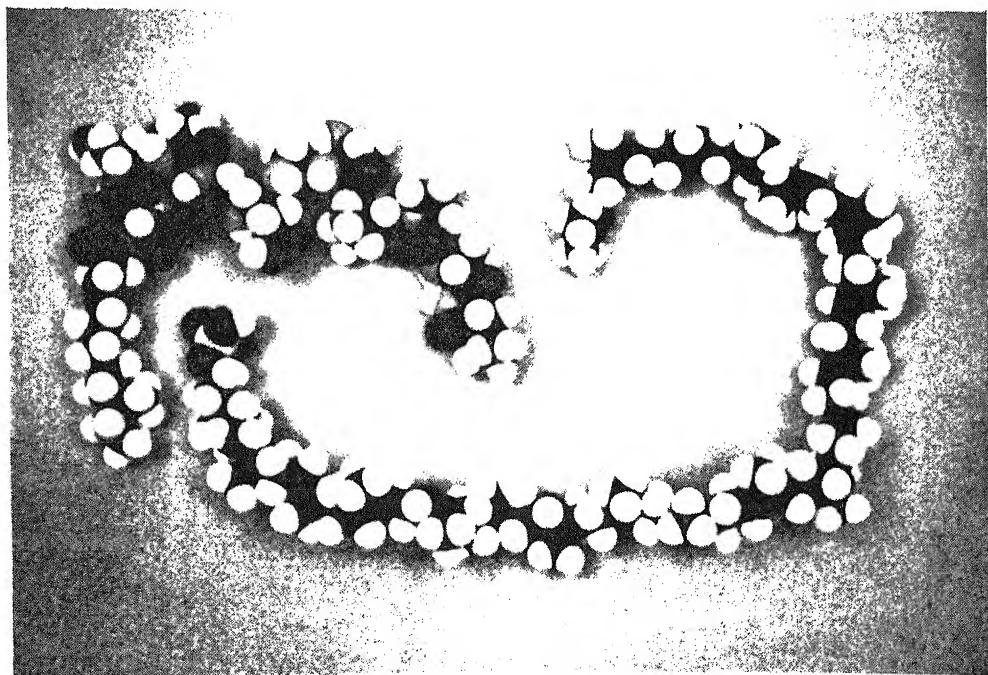


Figure 5. Site of interaction between amphomyein and Dol-P. A space filling model for amphomyein (upper) as well as for Dol-P (lower) have been constructed. The hydrophobic domain of Dol-P is for the anchorage to the membrane and the hydrophilic site acts as an

fold molar excess of EDTA immediately dissociated the complex (data not shown).

The  $\text{Ca}^{2+}$  dependent complex formation between Dol-P and amphotycin raises the question about its potential use to locate Dol-P in biologically active membranes. Simple kinetic measurements (figure 4) is in favour of Dol-P being present in the cytoplasmic site of the microsomal membranes. This observation is supported by the facts that (i) in absence of unlabelled amphotycin the binding of  $[\text{H}]-\text{amphotycin}$  to the oviduct microsomes is almost quantitative and (ii) the increasingly less binding in presence of excess unlabelled amphotycin. The quantitative reversal of  $[\text{H}]-\text{amphotycin}$  binding to the oviduct membranes, however, could not be detected even at the highest concentration of amphotycin used here. This may partially account for a non-specific interaction with other membrane phospholipids or 50% of the membranes formed wrong-side out vesicles. The probable site of interaction between these two molecules is explained in the space filling model (figure 5) but how  $\text{Ca}^{2+}$  plays a central role in holding the two molecules together needs to be investigated.

## References

- Cherjee, D. K., Scher, M. G. and Waechter, C. J. (1981) *Biochemistry*, **20**, 1561.
- Bodanszky, M., Sigler, G. F. and Bodanszky, A. (1973) *J. Am. Chem. Soc.*, **95**, 2352.
- Donner, W. M. and Laskey, R. A. (1974) *Eur. J. Biochem.*, **46**, 83.
- DeLuca, L. M., Fort-Coutaz, J., Silverman-Jones, C. S. and Roller, P. R. (1977) *J. Biol. Chem.*, **252**, 2575.
- Elbein, M. C., Gafford, J. T. and Elbein, A. D. (1977) *J. Biol. Chem.*, **252**, 7431.
- Elbein, M. C., Gafford, J. T. and Elbein, A. D. (1978) *Arch. Biochem. Biophys.*, **191**, 698.
- Finemann, B., Kaplan, M. A., Muir, R. D. and Hooper, I. R. (1953) *Antibiot. Chemother.*, **3**, 1239.
- Hoover, J. A. and Lennarz, W. J. (1978) *J. Biol. Chem.*, **254**, 9237.
- Lehman, F. W. (1977) *Biochem. Soc. Trans.*, **5**, 1223.
- Mont, L. A., Davidson, S. K. and Golemboski, D. B. (1983) *Arch. Biochem. Biophys.*, **226**, 347.
- Ng, M. S., Spencer, J. P. and Elbein, A. D. (1978a) *Biochem. Biophys. Res. Commun.*, **82**, 568.
- Ng, M. S., Spencer, J. P. and Elbein, A. D. (1978b) *J. Biol. Chem.*, **253**, 8860.
- Ng, M. S. and Elbein, A. D. (1979) *Arch. Biochem. Biophys.*, **198**, 304.
- Kornfeld, R. and Kornfeld, S. (1985) *Annu. Rev. Biochem.*, **54**, 631.
- Marasamy, R. and Symons, R. H. (1979) *Analyt. Biochem.*, **95**, 359.
- McCas, J. J. and Waechter, C. J. (1976) *Mol. Cell Biochem.*, **11**, 67.
- Prodi, A. J. and Leloir, L. F. (1979) *Biochim. Biophys. Acta.*, **559**, 1.
- Ueda, Y. and DeLuca, L. M. (1981) *Biochem. J.*, **200**, 529.
- Unger, M. D., Sultzman, L. A. and Robbins, P. W. (1980) *Cell*, **21**, 385.
- Winer, S. M. and Lester, R. L. (1972) *J. Bacteriol.*, **109**, 81.
- Wuck, D. K. and Lennarz, W. J. (1977) *J. Biol. Chem.*, **252**, 1007.
- Yatsuki, A., Kohno, K. and Tamura, G. (1975) *Agric. Biol. Chem.*, **39**, 2089.
- Yamaoka, H., Iwai, Y., Oiwa, R., Shinohara, S., Shimizu, S., Oka, T. and Omura, S. (1977) *Biochim. Biophys. Acta.*, **497**, 633.
- Yamaoka, H., Oiwa, R., Matsukura, S. and Omura, S. (1979) *Biochem. Biophys. Res. Commun.*, **86**, 902.
- Wacz, J. S. and Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun.*, **65**, 248.
- Yoshiaki, A. and Kornfeld, S. (1983) *J. Biol. Chem.*, **258**, 2808.
- Waechter, C. J. and Lennarz, W. J. (1976) *Annu. Rev. Biochem.*, **45**, 95.
- Waechter, C. J. and Harford, J. B. (1977) *Arch. Biochem. Biophys.*, **181**, 185.
- Yamashita, K., Veda, I. and Kobata, A. (1983) *J. Biol. Chem.*, **258**, 14144.



## Are the proteinase inhibitory activities in lenticular tissues real?

S. SWAMINATHAN and T. N. PATTABIRAMAN

Department of Biochemistry, Kasturba Medical College, Manipal 576 119, India

**Abstract.** The possibility of proteinase inhibitory activities in lenses measured with synthetic substrates being spurious, due to the effective competition of lens proteins as substrates for the target enzymes, was investigated. Goat, sheep and human cataractous lens proteins were found to be poor substrates for trypsin, elastase and papain compared to casein or bovine serum albumin. Further, the inhibition of elastase catalyzed hydrolysis of succinyl trialanyl *p*-nitroanilide by casein (500  $\mu$ g, 53%) and albumin (500  $\mu$ g, 49%) and of trypsin-catalyzed hydrolysis of benzoyl arginine *p*-nitroanilide by albumin (1 mg, 24%) were significant only at high protein concentrations. These data indicated that the relatively high antielastase and antitryptic activities observed in human cataractous lenses were real. On the other hand, coincident lens protein hydrolysis elevating the true antitryptic and antielastase activities in goat and sheep lenses (that have low activities) could not be ruled out. The lesser papain inhibitory activities observed in lenses when albumin was used as substrate compared to activities with benzoyl arginine *p*-nitroanilide as substrate, appeared to be partly due to lens protein hydrolysis masking the actual inhibition in the former method. Preincubation of goat, sheep and human lens extracts with trypsin for 1 h resulted in complete loss of antitryptic and antielastase activity except in the case of human lens antielastase activity which underwent 50% loss. Papain inhibitory activity was fully stable. Similar papain treatment caused loss of 80-100% of antielastase activity and 45-55% loss of antitryptic activity.

**Keywords.** Proteinase inhibitor; lenticular tissue; antielastase activity; antitryptic activity.

### Introduction

Tse and Ortwerth (1980) demonstrated antitryptic activity in mammalian lenses and suggested that alteration in the activity could be a determinant factor for cataractogenesis (Tse and Ortwerth, 1980a,b). Recently, the presence of factors capable of inhibiting trypsin, elastase and chymotrypsin in several mammalian lenses was reported from this laboratory (Swaminathan *et al.*, 1986). While the activities were manifested with synthetic substrates in the assay systems, with protein substrates like casein and albumin, the inhibitory activities could not be detected in the lenticular tissues (Swaminathan and Pattabiraman, 1986a). It was also observed that papain inhibitory activity was much higher in lens when the assay was performed with benzoyl arginine *p*-nitroanilide as substrate rather than with bovine serum albumin (BSA) or casein (Swaminathan and Pattabiraman, 1986b).

The inhibitory activities in the lenses were generally low requiring deployment of lens proteins several folds higher than the target enzymes to measure inhibition. Heat treatment of the normal lens extracts enhanced the proteinase inhibitory activities whereas preincubation with target enzymes decreased or abolished the activities (Swaminathan and Pattabiraman, 1986a,b). Further, magnitude of inhibition was linear with respect to lens protein concentration only over a limited range. These

data taken together, suggested that the inhibitory activities observed could be artifactual, in that the lens proteins by serving as substrates could competitively diminish the hydrolysis of synthetic substrates. This will cause an apparent inhibition and the increase in inhibitory activity observed with heat treated lens extracts could merely reflect better binding and hydrolysis of lens proteins.

The present study was undertaken to evaluate this possibility. We approached the problem by determining (i) the effect of protein substrates on the hydrolysis of amides by target enzymes, (ii) the correlation of the efficacy of lens proteins (native and denatured) as substrates for proteinases and their inhibitory potencies and (iii) the alteration of inhibitory activities in lens extract on digestion with different proteinases. The results are presented and evaluated in this communication.

## Materials and methods

The sources of human cataractous lenses, sheep and goat lenses were mentioned earlier (Swaminathan *et al.*, 1986). Bovine trypsin (twice crystallized) and porcine elastase (twice crystallized) were purchased from Worthington Biochemical Corporation, Freehold, New Jersey, USA. Pepsin (porcine, crystallized), papain (twice crystallized),  $\alpha$ -N-benzoyl DL-arginine *p*-nitroanilide (BAPNA), succinyl tri-L-alanyl *p*-nitroanilide (STANA), benzamidine and BSA were from Sigma Chemical Company, St. Louis, Missouri, USA. Casein was from British Drug House Chemical Limited, Poole, England. All other reagents were analytical grade commercial chemicals.

Goat, sheep and human lens extracts were prepared in 20 mM sodium phosphate buffer, pH 7.6 as described earlier (Swaminathan and Pattabiraman, 1986b). For experiments involving the determination of antitryptic and antielastase activities the lens extracts were prepared in 20 mM Tris-HCl buffer pH 7.6 (Swaminathan *et al.*, 1986). The lens extracts when necessary were subjected to heat treatment at 60°C for 15 min prior to use. Albumin and casein solutions were prepared in Tris-HCl buffer (0.1 M), pH 7.6 at concentrations of 10 mg/ml. Heat denatured albumin was prepared by subjecting the solution to heat treatment at 100°C for 2 min. Similarly, casein was denatured by heating at 100°C for 10 min.

## Assay of proteinases and inhibitors

Trypsin and elastase were assayed using BAPNA and STANA respectively as substrates (Swaminathan *et al.*, 1986). Under the assay conditions (37°C, 15 min, pH 7.6) trypsin (16.0  $\mu$ g protein) and elastase (2.0  $\mu$ g protein) released *p*-nitroaniline equivalent to an absorbance of 0.6 ( $\lambda$ 410). For routine purposes, papain was assayed using either BSA or BAPNA (Swaminathan and Pattabiraman, 1986b). Under the assay conditions with albumin (5 mg, 12.5  $\mu$ g papain, 15 min, 37°C, pH 7.6) papain liberated trichloroacetic acid (TCA) soluble fragments equivalent to an absorbance of 0.6 ( $\lambda$ 540). With BAPNA as substrate (5  $\mu$ mol) papain (125  $\mu$ g) liberated under the assay conditions (30 min, pH 7.6, 37°C) *p*-nitroaniline equivalent

The assays were based on the method of Kunitz (1947). The system consisted of lens extracts (native or denatured), 40  $\mu\text{mol}$  of sodium phosphate buffer pH 7.6 and the enzyme (trypsin 16.0  $\mu\text{g}$ , elastase 2.0  $\mu\text{g}$  or papain 12.5  $\mu\text{g}$ ) in a volume of 2.0 ml. After 15 min incubation at 37°C, the reaction was arrested by the addition of 3.0 ml of 5% TCA. The suspension was centrifuged (2500 rpm) and the extent of lens protein degradation was estimated by determining the TCA soluble fragments in 1 ml fractions of the supernatant by the method of Lowry *et al.* (1951). To study pepsin digestion, 3.7  $\mu\text{g}$  of the enzyme was incubated with the lens extract in the presence of 40  $\mu\text{mol}$  of HCl-KCl buffer pH 2.0. The processing was otherwise similar.

*Effect of treatment of lens proteins with proteinases on their inhibitory activities*

Lens extracts were preincubated with different proteinases individually for definite time intervals at 37°C. The residual inhibitory activities were determined according to the methods described above. In the cases of preincubation with the target enzyme itself, inactivation of the enzyme, prior to assay of inhibitory activities did not arise. The enzyme was treated with the lens extracts at pH 7.6 for definite time followed by the addition of the substrate. Control system with enzyme alone was run concurrently. In other cases controls without the digesting proteinases were run simultaneously.

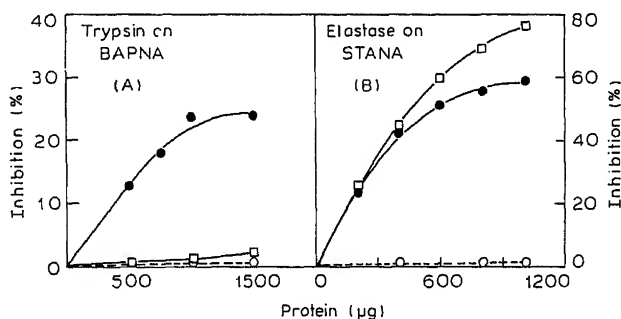
*Pepsin treatment:* Lens extracts (5–15 mg protein) were incubated with pepsin (3.7  $\mu\text{g}$ ) at pH 2.0 (0.04 M HCl-KCl) in a volume of 1.0 ml. The reaction was stopped by adding 2.0 ml of 0.2 M Tris-HCl buffer pH 7.6. Aliquots were assayed for residual antitryptic, antielastase and antipapain activities.

*Papain treatment:* Lens extracts (0.15–2.20 mg protein) were treated with papain (12.5  $\mu\text{g}$ ) in the presence of 4  $\mu\text{mol}$  of EDTA, 9  $\mu\text{mol}$  of  $\beta$ -mercaptoethanol and 40  $\mu\text{mol}$  of Tris-HCl buffer pH 7.6. After incubation, 10  $\mu\text{mol}$  of  $\text{CaCl}_2$  was added (final volume 1.8 ml) and aliquots were assayed for antitryptic and antielastase activities.

*Trypsin treatment:* Lens extracts (0.08–1.40 mg protein) were incubated with 16  $\mu\text{g}$  of trypsin in the presence of 40  $\mu\text{mol}$  of buffer pH 7.6 (Tris-HCl for antielastase and sodium phosphate for antipapain determinations) in a volume of 1.8 ml. Aliquots were taken and directly assayed for residual antielastase activity. For measurement of antipapain activity with BAPNA as substrate 5 mg of benzamidine was added (final volume 1.8 ml) to completely inactivate trypsin and aliquots were then used for the estimation. Benzamidine at the concentration used did not affect papain action and also it did not alter the papain inhibitory activity in lens extracts.

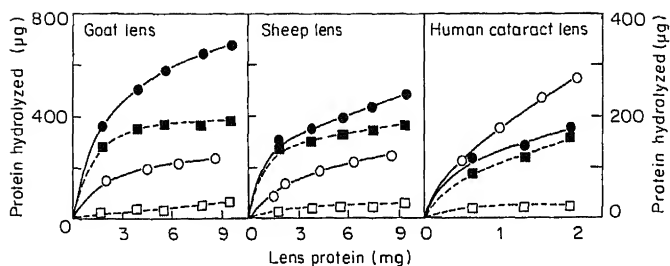
Protein was determined by the method of Lowry *et al.* (1951) using BSA as standard.

Figure 1 depicts the action of BSA and casein on the amidolysis of BAPNA by trypsin and of STANA by elastase. The concentrations of the proteins used were in the same range in which lens proteins were employed for routine inhibitory assays. Hydrolysis of BAPNA was virtually unaffected by casein (figure 1A). Heat denatured casein also behaved similarly (data not shown). While BSA did not reduce the action of trypsin on BAPNA, heat treated BSA showed moderate reduction of *p*-nitroaniline release. Elastase catalyzed hydrolysis of STANA was more sensitive to the inclusion of proteins in the assay medium. Casein was effective in diminishing STANA hydrolysis (figure 1B). Heat treated casein however did not exhibit any enhanced action (data not shown). While BSA did not reduce elastase action, heat denatured BSA was moderately effective in reducing STANA hydrolysis. Comparison of the data with that of lens proteins showed that 1 mg of casein, heat treated BSA, goat lens protein, sheep lens protein and human cataract lens protein diminished elastase activity on STANA to the extents of 2.33  $\mu$ g, 2.50  $\mu$ g, 0.5–1.1  $\mu$ g ( $n=10$ ), 0.5–2.35  $\mu$ g ( $n=10$ ) and 8.3–25.1  $\mu$ g ( $n=10$ ) equivalents of elastase, respectively. Similarly, 1 mg of heat treated BSA, goat, sheep and human lens proteins inhibited 3.73  $\mu$ g, 1.87–4.0  $\mu$ g ( $n=10$ ), 1.06–6.6  $\mu$ g ( $n=10$ ) and 12.0–42.9  $\mu$ g, ( $n=10$ ) of trypsin in terms of suppression of BAPNA hydrolysis. Similar studies on papain catalyzed hydrolysis of BAPNA showed that 1 mg of BSA, goat, sheep and human lens proteins (all heat denatured) will suppress papain action to the tune of 8.4  $\mu$ g, 24.5  $\mu$ g ( $n=5$ ), 25–42.0  $\mu$ g ( $n=5$ ) and 45–90  $\mu$ g ( $n=5$ ) of the enzyme, respectively. The values are based on the linear ranges of inhibition.



**Figure 1.** Diminution of tryptic and elastase activity on synthetic substrates by casein and albumin. (□), Casein; (○), albumin; (●), heat treated albumin. Other details are given under 'materials and methods'.

If observed 'inhibitions' are due to coincident hydrolysis of protein substrates by target enzymes, it should be expected that human cataractous lens proteins will be efficient substrates for the proteinases. The extents of hydrolysis of lens proteins by different proteinases in the range of concentrations used for inhibition assays are presented in figure 2. For comparison, the action of pepsin is also shown. Under the assay conditions, tryptic digestion was far more effective than elastase action with all



**Figure 2.** Hydrolysis of lens proteins by proteinases. (●), Trypsin; (■), papain; (○), pepsin; (□), elastase. Other details are given under 'materials and methods'.

of human cataract lenses alone pepsin was more effective than trypsin. Nevertheless, the lens proteins were found to be poor substrates for proteinases compared to casein or albumin. The relative rates of hydrolysis based on initial rates, at 1 mg protein level for tryptic action, was calculated to be 20.2% for goat lens, 15.4% for sheep lens, 17.3% for human cataract lens and 40.4% for BSA compared to casein. Similarly, the relative values for goat, sheep and human lens for elastase were respectively 20.1, 24.2 and 30.3% and for papain, 50.0, 44.4 and 38.9%. The relative values for pepsin hydrolysis (with hemoglobin as 100%) for the 3 lens proteins were 13.0, 11.1 and 37.0%, respectively.

The lens extracts were subjected to heat treatment (60°C, 15 min) and were then used as substrates for the proteinases. The magnitudes of increase in proteolytic activities are shown in table 1. The changes in proteinase inhibitory activities on heat treatment are also shown. Heat treatment enhanced hydrolysis by trypsin, elastase and papain in all cases excepting the action of elastase on human lens. Heat denaturation of lens proteins had no effect on peptic action. No correlation between increase in proteolysis and enhancement of inhibitory activity could be found in individual cases except that heat treatment of human cataract lens extracts neither accentuated its digestion by elastase nor affected its antielastase activity. The data suggest that increased susceptibility of lens proteins to proteinase digestion need not reflect the observed increase in inhibitory activity, on heat treatment.

The lens extracts were treated with different proteinases and the residual proteinase inhibitory activities were estimated in goat, sheep and human lenses.

**Table 1.** Effect of heat treatment of lens extracts on their proteolysis and proteinase inhibitory activities.

Proteinase	Increase in proteolysis (%)			Increase in proteinase inhibitory activity (%)		
	Goat	Sheep	Human	Goat	Sheep	Human
Trypsin	49	123	71	180	140	0
Elastase	233	166	0	74	90	0
Papain	76	106	83	301	307	295
Pepsin	0	0	0	—	—	—

Lens extracts were subjected to heat treatment at 60°C for 15 min. Values



**Table 2.** Proteinase inhibitory activities in lens extracts after prior treatment with proteinases.

Digestion system	Residual activity (%)		
	Goat lens	Sheep lens	Human lens
<i>Elastase inhibition</i>			
Trypsin, 30 min	0.0	0.0	48.6
Papain, 30 min	28.0	26.5	7.5
Papain, 60 min	11.5	19.5	0.0
Pepsin, 60 min	78.0	81.0	87.0
Pepsin, 120 min	69.0	65.0	81.0
Elastase, 60 min	0.0	0.0	75.0
<i>Trypsin inhibition</i>			
Papain, 30 min	75.0	72.5	62.0
Papain, 60 min	56.0	44.5	52.0
Pepsin, 60 min	80.0	89.0	91.0
Pepsin, 120 min	80.0	83.0	91.0
Trypsin, 60 min	0.0	0.0	0.0
<i>Papain inhibition</i>			
Trypsin, 60 min	104.0	100.0	85.4
Trypsin, 120 min	104.0	95.0	63.3
Pepsin, 60 min	100.0	104.0	100.0
Pepsin, 120 min	104.0	100.0	90.0
Papain <sup>a</sup> , 60 min	0.0	0.0	0.0
Papain <sup>b</sup> , 60 min	100.0	104.0	100.0

<sup>a</sup>Inhibitory activity was measured with BAPNA as substrate.

<sup>b</sup>Inhibitory activity was measured with BSA as substrate.

Other details are given under 'materials and methods'.

Typical data are shown in table 2. Digestion with trypsin for 30 min or with elastase for 60 min completely abolished the antielastase activity in goat and sheep lenses whereas the human lens retained antielastase activity to the extent of 50% following trypsin treatment and 75% following elastase treatment. Papain treatment also caused considerable loss of antielastase activity. Pepsin was not very effective in this respect. While preincubation with trypsin resulted in rapid loss of antitryptic activity, papain and pepsin were less effective in this regard. Papain inhibitory activity was highly resistant to the action of trypsin and pepsin. Preincubation studies with papain with reference to papain inhibitory activity differed depending on the assay procedure. In the assay with BAPNA as substrate, papain inhibitory activity was completely abolished on pretreatment with the target enzyme whereas by the BSA assay, no loss of inhibitory activity could be observed. This appears to be more of a reflection of the enzyme concentration used. In the BAPNA assay method the amount of enzyme used is nearly an order of magnitude higher compared to the quantity used in the BSA method. Since elastase showed relatively weak proteinase activity under the assay conditions (9–16 times lower than tryptic activity on different lens proteins), preincubation effect on inhibitory activities other than the target enzyme was not studied. Correlation of the data in table 2 with magnitudes of hydro-

only factor responsible for apparent inhibition observed, it should be expected that trypsin treatment should abolish all the 3 proteinase inhibitory activities.

It has been shown earlier (Swaminathan and Pattabiraman, 1986b), the papain inhibitory activity in heat treated lens extracts was much higher when BAPNA was used as substrate rather than BSA. Papain inhibitory activities were compared in this study using both the substrates, but with identical concentration of the enzyme (17  $\mu\text{g}$ ). In this modified BAPNA assay system, the time of incubation was increased to 5 h so that the control enzymatic activity ( $\lambda$  410, 0.5) was comparable to the value obtained in the earlier described assay (Papain 125  $\mu\text{g}$ ; assay time 30 min). The papain inhibitory activities estimated under the different assay conditions are shown in table 3. The data indicate that the wide differences observed between the values by the BSA method and BAPNA method were narrowed down when the concentration of the enzyme used in the two methods were the same. To evaluate the real magnitude of papain inhibition with BSA as substrate, a sequential experiment with goat lens extract was performed and the results are shown in table 4. Difference between system A and B will be an index of the measured inhibitory activity. Preincubation of the lens extract for 10 min with papain followed by addition of substrate and incubation for further 15 min did not alter the value of inhibition (A - C). Incubation of the lens extracts with papain for 10 min and 25 min represented by D and E indicate the hydrolysis of lens proteins by papain. The data suggest that while the apparent inhibition is 0.10, the actual inhibition could be a maximum of 0.20 (A - B + D) for

**Table 3.** Papain inhibitory activities in lens extracts measured with BSA and BAPNA as substrates.

Lens	Papain ( $\mu\text{g}$ ) inhibited per mg lens protein		
	BSA	BAPNA <sup>a</sup>	BAPNA <sup>b</sup>
Human-1	4.85	17.20	54.0
Human-2	3.76	9.15	39.0
Human-3	3.04	5.11	35.0
Goat-1	2.74	8.90	25.0
Goat-2	2.06	5.02	22.0
Goat-3	1.78	2.81	18.0

<sup>a</sup>Assay conditions were, 17  $\mu\text{g}$  papain, 5 h assay time at 37°C.

<sup>b</sup>Assay conditions were, 125  $\mu\text{g}$  papain, 30 min assay time at 37°C.

**Table 4.** Sequential studies on papain inhibition with goat lens extract.

Assay condition	$\lambda$ 540
A. Papain (12.5 $\mu\text{g}$ ) + BSA (5 mg): 15 min	0.58
B. Papain + BSA + Lens extract (0.54 mg): 15 min	0.48

proteins.

Papain inhibitory activity was found to be still lower when casein instead of BSA was used as substrate in the assay system (Swaminathan and Pattabiraman, 1986b). The effect of heat treatment of the lens extracts on inhibitory activity against papain was studied using BSA and casein as substrates. While the heat treatment increased the papain inhibitory activity with BSA as substrate, in goat lens (1.92–3.05 fold,  $n=6$ ) and sheep lens (2.18–3.53 fold,  $n=6$ ), in human cataractous lens the changes were marginal (0.95–1.06 fold,  $n=6$ ). In contrast, with casein as substrate the papain inhibitory activity in goat lens and sheep lens were completely abolished whereas in human lens the activity remained virtually unchanged.

## Discussion

Gaudin and Stevens (1974) showed that  $\alpha$ -crystallin a major lens protein can inhibit trypsin and chymotrypsin. It was shown later that trypsin can degrade  $\alpha$ -crystallin (Siezen and Hoenders, 1977). The present observations that lens proteins undergo hydrolysis, although to a limited degree, and that heat treatment of lens extract enhances digestion by proteinases in most cases would suggest that the observed antielastase, antitryptic and antipapain activities in lens extracts can be spurious. The observed inhibitions thus, would merely be due to decreased hydrolysis of the synthetic substrates used in the assays, due to effective competition of lens protein for the target enzymes as substrates. The reduction in elastase-catalyzed STANA hydrolysis by casein and of the elastase-catalyzed STANA hydrolysis and tryptic action on BAPNA by heat denatured albumin enforce this view.

However, a closer scrutiny of the available data counteracts this possibility at least partially. Even though casein and heat treated albumin reduce STANA hydrolysis by elastase, the lens proteins themselves were found to be poor substrates for elastase emphasizing that the analogy is not wholly valid. Further, with human cataract lenses which had high antielastase activity about 20–60  $\mu\text{g}$  lens protein caused around 25% inhibition of the enzyme whereas at these levels casein and albumin had negligible effect. Even though the inhibitory activity in human lenses was higher by an order of magnitude compared to goat and sheep lenses, the rates of digestion of the 3 lens proteins by elastase did not differ significantly. While preincubation with elastase caused complete inactivation of antielastase activity in goat and sheep lenses, it was observed earlier that in the case of human lenses the decrease in inhibitory activity is marginal (Swaminathan and Pattabiraman, 1986a). The relative rates of digestion of lens proteins also do not parallel the magnitudes of loss of antielastase activity on proteinase treatment. The data suggest that the observed antielastase activity even in goat and sheep lenses cannot exclusively be due to competition of lens proteins as substrates.

With reference to antitryptic activity also, the available data lead to similar conclusions. Casein a good substrate for trypsin did not reduce BAPNA hydrolysis and hence it is not possible to expect that the lens proteins that are poor substrates could efficiently reduce tryptic action on BAPNA. Further, while heat treatment of lens extracts made them better substrates for trypsin in all cases, increase in inhibitory activity was observed only with goat and sheep lenses. While papain digested human

antitryptic activity whereas, trypsin completely abolished the activity on preincubation.

In regard to thiol proteinase inhibitory activity in lens, the possibility of substrate competition resulting in apparent inhibition seems rather remote. Heat treatment of the lens extracts increased papain inhibitory activity measured with BSA as substrate, only in goat and sheep lenses but not in human lenses. However, proteolysis by papain was found to be enhanced by heat treatment in all the 3 lenses. If competitive proteolysis is responsible for inhibitory effect, it should be expected that heat treatment should actually decrease the observed inhibition in this assay system. Interestingly, papain inhibitory activity, measured with casein as substrate, which was much lower than the values observed with BSA (Swaminathan and Pattabiraman, 1986b) was abolished in goat and sheep lenses on heat treatment. The variations in papain inhibitory activities measured with different substrates and the differential behaviour of the activity on heat treatment between normal and cataractous lenses could be due to the presence of more than one papain inhibitor and their altered proportions in cataractous lenses. The importance of differences in the affinity of substrates for papain can also be a causative factor.

The earlier observation on the higher papain inhibitory activity (by an order of magnitude) observed with BAPNA as substrate compared to the values with BSA is now shown to be mainly due to differential assay conditions. At present, we do not have an explanation as to why inhibitory activity measured with BAPNA as substrate is dependant on enzyme concentration. However, the higher inhibitory activities observed with synthetic substrate compared to BSA system even at comparable concentration of enzyme can be partially explained as due to the limitation of the BSA assay method. In this procedure the digestion of lens protein coincident to inhibition, tends to mask the inhibitory activity. Failure to observe antitryptic and antielastase activities in lens extracts when casein or albumin is used as substrate (Swaminathan and Pattabiraman, 1986a) might be due to this limitation. In this context it is worthwhile to note that Tse and Ortwerth (1980b) observed antitryptic activity in lenses using azocasein as substrate. In their assay procedure it is the 'solubilized' dye that was measured as an index of proteolysis and inhibition.

Collation of the available information indicates that antielastase and antitryptic activities measured in human cataractous lenses are real and coincident lens protein hydrolysis has no effect on the observed values. With sheep and goat lenses, which have relatively low serine proteinase inhibitory activities, substrate competition effect contributing to observed inhibition cannot be completely ruled out. In regard to papain inhibition the magnitude of observed inhibition with BSA as substrate will be an underestimation due to interfering lens protein hydrolysis.

## Acknowledgements

This work was supported by a grant from Council of Scientific and Industrial Research, New Delhi. The first author is a recipient of the Council of Scientific and Industrial Research, New Delhi, senior research fellowship.

## References

Siezen, R. J. and Hoenders, H. J. (1977) *FEBS Lett.*, **80**, 75.

Swaminathan, S., Chandrasekher, G., Venkataraman, A. and Pattabiraman, T. N. (1986) *Biochem. Med. Metabol. Biol.*, **35**, 184.

Swaminathan, S. and Pattabiraman, T. N. (1986a) *Biochem. Med. Metab. Biol.*, **36**, 313.

Swaminathan, S. and Pattabiraman, T. N. (1986b) *Indian J. Clin. Biochem.*, **1**, 30.

Tse, S. S. and Ortwerth, B. J. (1980a) *Exp. Eye Res.*, **31**, 313.

Tse, S. S. and Ortwerth, B. J. (1980b) *Exp. Eye Res.*, **31**, 411.

## Physicochemical properties and binding-site amino acid residues of galactoside-binding protein of human placenta

MADHUSOODHAN P. NAMBIAR, DEBKUMAR BASU\* and  
P. S. APPUKUTTAN

Neurochemistry Department, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum 695 011, India

**Abstract.** The galactose-binding lectin of human placenta has been purified to homogeneity by affinity chromatography on asialo-fetuin column. The protein, extractable from the tissue only with lactose is apparently membrane-bound. Molecular weight determination of native protein and subunit indicated a dimer of 13.4 kDa subunits. Inhibition of haemagglutination with various saccharides indicate that thiodigalactoside is the best inhibitor followed by lactose. However, *p*-nitrophenyl- and 1-O-methyl derivatives of galactose showed that  $\alpha$ -anomers inhibited slightly better than  $\beta$ -anomer. Modification of amino acid residues indicated involvement of arginine, lysine and histidine residues at the saccharide-binding site. Cysteine residue modification also abolished haemagglutinating activity. Amino acid composition of the lectin is also presented.

**Keywords.** Human placenta; galactose-binding lectin; binding-site amino acids; chemical modification.

### Introduction

The role of carbohydrates in cellular recognition is well documented. It has become increasingly apparent that cell surface carbohydrates are intimately involved in lymphocyte 'homing', tumour invasiveness, trophoblast implantation and inter-cellular adhesion (Ashwell, 1977). The recently emerged field of carbohydrate-specific binding proteins, particularly, the galactose-binding one in hepatic tissue has been reviewed by Ashwell and Harford (1982). Barondes (1984) has reviewed soluble galactose-binding protein from several animal tissues. Some lectins of certain animal species are also developmentally regulated. These  $\beta$ -galactose-binding lectins are isolated in mono-, di- or oligomeric forms (Carding *et al.*, 1985). The predominant lectins contain subunits in the range 13-26 kDa. A free SH-group is an essential feature of these lectins. The lectins are generally extracted from frozen or fresh tissue in presence of lactose and require no metal ions, with the exception of human hepatic lectin (Baenziger and Maynard, 1980). Recently, Hirabayashi and Kasai (1984) have reported a  $\beta$ -galactoside-binding lectin from human placenta.

In this communication we report the isolation and detailed physicochemical studies of the same lectin from normal human placenta. We have also examined the amino acids involved at the sugar-binding site by their chemical modification using specific reagents.

\*To whom all correspondence should be addressed.

Galactopyranosylamine, thiodigalactoside, galactosamine, melibiose, raffinose, stachyose, 1-O-methyl- $\alpha$ - and  $\beta$ -D-galactose, 1-O-methyl- $\alpha$ -D-glucose, chitin, 1-O-methyl- $\alpha$ -D-mannose, wheat germ, *p*-nitrophenyl derivatives of galactose, N-acetyl-midazole, maleic anhydride, molecular weight markers (Aldolase, soybean trypsin inhibitor, ovalbumin, bovine serum albumin), citraconic anhydride, fetuin, *p*-hydroxymercuribenzoate (pHMB), dithionitrobenzoic acid (DTNB), sodium dodecyl sulphate (SDS) and trinitrobenzenesulphonic acid (TNBS) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. 2-Hydroxynitrobenzyl bromide, phenyl glyoxal hydrate, 2-methoxynitrobenzyl bromide, diethyl pyrocarbonate, 1,2-cyclohexanedione and N-ethyl morpholine were obtained from Fluka, Buchs, Switzerland. Sephadex and Sepharose were the products of Pharmacia Fine Chemicals, Sweden. Biogel P-60 and P-100 were purchased from BioRad Laboratories, USA. All other reagents were of analytical grades and obtained from local sources.

Normal full-term placenta tissue were collected from Trivandrum Medical College, in ice. The tissue was cleaned of blood and other adhering tissue material and kept frozen at  $-20^{\circ}\text{C}$  until use. The trophoblasts were separated from freshly collected placenta and kept overnight at  $0^{\circ}\text{C}$  in ice. All the operations were carried out at  $0-4^{\circ}\text{C}$  unless otherwise mentioned. The centrifugation was done with SORVALL-RC-5B centrifuge. Frozen or fresh tissue was used for the isolation of galactose-binding protein. The homogeneous protein from both the types of tissue were found to be electrophoretically and immunologically identical. The buffer used for all the experiments was 20 mM phosphate-150 mM sodium chloride-4 mM mercaptoethanol, pH 7.2 (phosphate buffered saline, PBS) unless otherwise mentioned.

Galactose-binding protein was isolated according to Hirabayashi and Kasai (1984) with modification. One-hundred gram of tissue was homogenised with 500 ml of PBS, pH 7.2 in SORVALL Omnimixer at 25% of its maximum speed for 2 min. The homogenate was centrifuged at 6000 *g* for 20 min. The pellet was extracted twice with 100 ml of PBS, pH 7.2 containing 100 mM lactose for 30 min. After each extraction the supernatant was collected by centrifugation as above. From the combined lactose extracts, proteins were precipitated with solid ammonium sulphate (56 g/100 ml) and dissolved in minimum volume of PBS, pH 7.2. The clear solution was dialyzed against the same buffer with 2 changes for 18 h.

The dialyzed solution was applied on a ( $2 \times 18$  cm) asialofetuin-Sepharose column. The column was prepared according to deWaard *et al.* (1976). The column was equilibrated and washed with PBS, pH 7.2 until the effluent had  $A_{280}$  below 0.02. The galactose-binding protein was eluted with the same buffer containing 20 mM lactose and 3 ml fractions were collected. The haemagglutinating activities of the dialyzed fractions were examined with trypsinised rabbit or human B + ve erythrocytes. The active fractions were pooled, dialyzed against PBS, pH 7.2 for 18 h and concentrated by ultrafiltration through Amicon PM-10 membrane.

Protein concentration was estimated by the method of Lowry *et al.* (1951) or by Bradford's protein assay (1976). Electrophoresis was carried out on polyacrylamide gel according to Davis (1964) and SDS polyacrylamide slab gel according to Laemmli (1970). The chemical modification of various amino acids were undertaken to obtain an idea of the sugar-binding region of the lectin. Amino groups were modified with TNBS (Fields, 1972), maleic anhydride (Butler *et al.*, 1969) and

tronic anhydride (Atassi and Habeeb, 1972). Sulphydryl group was estimated and modified with DTNB according to Habeeb (1972) and also with N-ethylmaleimide and pHMB (Riordian and Vallee, 1972). Tyrosine was titrated and modified with N-ethylimidazole (Riordian and Vallee, 1972). Tryptophan was modified with dimethyl sulphoxide/acetic acid/HCl (Savage and Fontana, 1977). Tryptophan was also modified with 2-hydroxy- and 2-methoxynitrobenzyl bromide (Riordian and Vallee, 1972). Arginine was modified with phenyl glyoxal hydrate (Takahashi, 1968), 1,2-cyclohexanedione (Smith, 1977) and histidine by the method of Miles (1977). Carbohydrate residues were modified by periodic acid (Spiro, 1966) and with periodate and sodium metaborohydride (Thorpe *et al.*, 1985). Total carbohydrate was estimated by phenol-sulphuric acid method (Dubois *et al.*, 1965). The molecular weight of the protein under undenaturing condition was carried out on Biogel P-100 in PBS, pH 7.2 containing 2.5 mM 2-mercaptoethanol according to Andrews (1965). Concanavalin A was purified and immobilised to Sepharose 4B by CNBr-methods of Porath as modified by Surolia *et al.* (1973). Wheat germ agglutinin (WGA) was purified on chitin column according to Bloch and Burger (1974). It was immobilised on Sepharose 4B by CNBr-method (Axen *et al.*, 1967). Castor bean agglutinin (RCA1) was purified and immobilised according to the method of Appukuttan *et al.* (1977). Kidney bean agglutinin (WBA) and Jack fruit seed lectin (Jacalin) were purified according to methods published earlier (Appukuttan and Basu, 1981; Sureshkumar *et al.*, 1982).

## Results and discussion

The affinity-eluted galactose-binding protein was homogeneous as confirmed by polyacrylamide gel electrophoresis (PAGE). The molecular weight of the undenatured lectin by gel filtration was found to be 26.9 kDa (figure 1). The subunit molecular weight of 13.4 kDa (figure 2) as revealed by SDS-PAGE was in agreement with the observation of Hirabayashi and Kasai (1984). These results suggest that the lectin is a dimer. We have not observed any molecular weight higher than 27 kDa when by gel filtration on Biogel P-60 or P-100. The lectin did not contain any  $\alpha$ -glucosidase activity.

The protein could agglutinate several native and trypsinised animal and normal human B+ve cells. It agglutinated native calf, pig, rat, mice and rabbit erythrocytes but not those of sheep, goat and dog. But after trypsinization of the cells it agglutinated rabbit, calf, pig, sheep, dog, rat, mice and normal human B+ve erythrocytes but not that of goat. Trypsinised human cells of A, B, AB and O groups were also agglutinated by placenta galactose-binding lectin. This lectin is non-mitogenic towards human lymphocytes.

The saccharide-inhibition studies of haemagglutination were carried out with trypsinised rabbit and human B+ve erythrocytes. The inhibition studies are shown in table 1. Thiodigalactoside was found to be the best inhibitor followed by lactose, also shown by Hirabayashi and Kasai (1984). Agglutination of trypsinised human cells was inhibited by 8-times lower concentration of thiodigalactoside as compared to rabbit cells. Similarly lactose concentration was 4-fold lower under identical conditions for human cells compared to rabbit cells. It has also been observed that



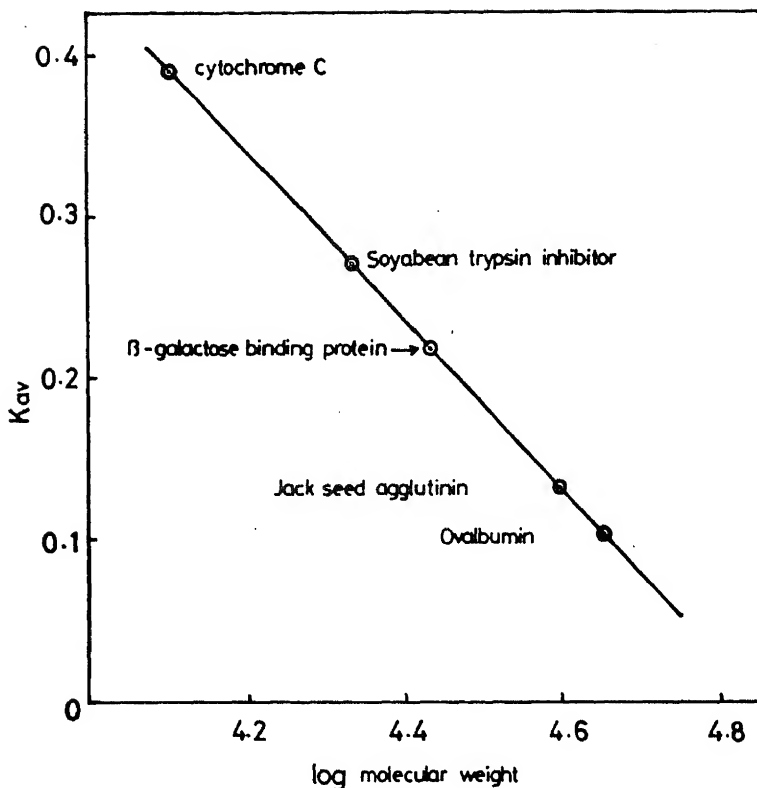
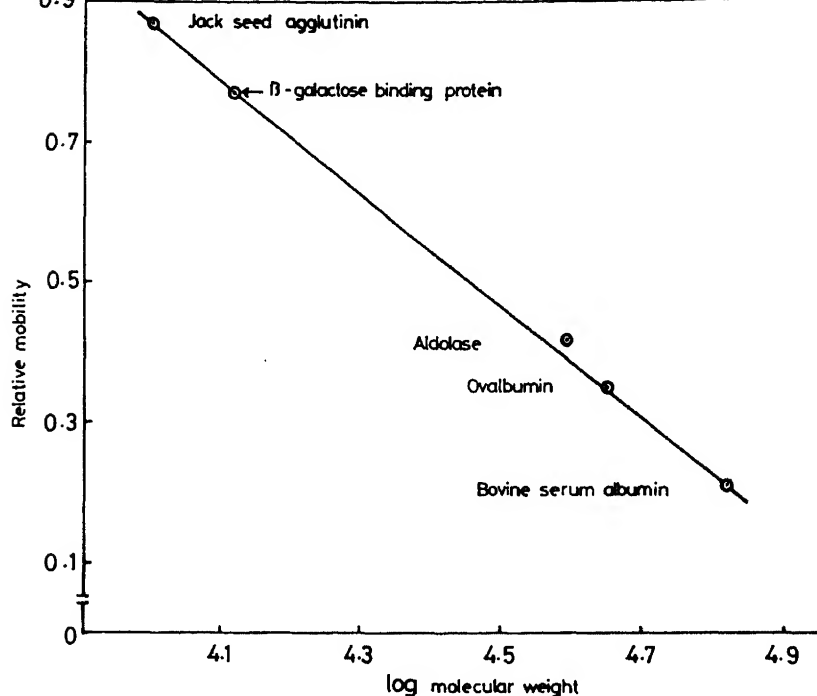


Figure 1. Gel filtration was carried out on Biogel P-100 ( $2 \times 78$  cm) in PBS, pH 7.2 containing 2 mM mercaptoethanol at  $25^\circ\text{C}$ . 2.5 ml fractions were collected at a flow rate of 12 ml/h. The standard proteins employed are cytochrome C, soybean trypsin inhibitor, Jack seed agglutinin (Sureshkumar *et al.*, 1982) and ovalbumin.

was found to be slightly better inhibitor than its  $\beta$ -anomer. But glucose or mannose did not inhibit agglutination with both the cell types. In the present study we have observed that the saccharide concentrations required for the inhibition of agglutination were much lower than reported by Hirabayashi and Kasai (1984). Our observations were repeated with several batches of tissue extracts. It has been observed with fresh or frozen tissue, as well as trophoblasts cells of normal tissue. We did not require any protease inhibitor addition during the isolation of the lectin. The lectin retained its agglutinating activity when kept in the presence of 1 mM mercaptoethanol in PBS for several weeks at a concentration of 1 mg/ml at  $0^\circ\text{C}$ . Protein concentration higher than 1 mg/ml resulted in aggregation after 3–4 days at  $0^\circ\text{C}$ . The lectin lost about 80% of its agglutinating activity within a week when kept frozen at  $-20^\circ\text{C}$ . But the cleaned, whole placenta tissue retained activity under similar conditions for 2 weeks. The agglutination of trypsinised human erythrocytes required 6-fold higher amount of lectin as compared to rabbit cells. The concentrations of inhibitory saccharides required for inhibiting agglutination were also lower in case of



**Figure 2.** SDS-Polyacrylamide gel (10% gel) was carried out according to Laemmli (1970). The standard proteins are Jack seed agglutinin, aldolase, ovalbumin and bovine serum albumin.

Ellman's method (Ellman, 1959) or even under denaturing condition as described by Habeeb (1972). Tryptophan residue could not be detected by the methods of Savage and Fontana (1977) or by spectral shift (Bencze and Schmid, 1957). Tyrosine residues were determined by O-acetylation with N-acetyl imidazole (Riordian and Vallee, 1972). The lectin contained 3 tyrosine residues. This was confirmed by aminoacid analysis (table 3). Total carbohydrate content estimated by phenol-sulphuric acid method (Dubois *et al.*, 1965) with galactose as standard was found to be 16.7%.

There is no information in the literature about the amino acids involved in the saccharide-binding site of  $\beta$ -galactose-binding protein. In order to get some insight into the binding site we have chemically modified a few amino acid residues. The glycoprotein did not contain any tryptophan residue. Similar observation has been made by Hirabayashi and Kasai (1984) from amino acid analysis. Tyrosine residues were found not to be involved in the saccharide binding. On the other hand arginine residue modification resulted in complete inhibition of agglutination. Histidine modification by diethyl pyrocarbonate showed inhibitory effect on agglutination. The amino group modification by various method had inhibitory effect. Similar results were obtained by SH-group modifications.

The oxidation of saccharide side chains by periodic acid had inhibitory effect. This

Saccharides	Minimum concentration (mM) required to inhibit twice the haemagglutinating amount of lectin	
	Trypsinised human B + ve cells	Trypsinised rabbit cells
Thiodigalactoside	0.019	0.16
Lactose	0.20	0.78
Lactitol	4	4
D-Galactose	25	50
Melibiose	12.5	50
Raffinose	25	100
Stachyose	12.5	25
D-Galactosamine	25	50
D-Galactopyranosylamine	0.39	6.25
N-Acetyl-D-galactosamine	25	100
1-O-Methyl- $\alpha$ -D-galactose	3.13	25
1-O-Methyl- $\beta$ -D-galactose	12.5	50
p-Nitrophenyl- $\alpha$ -D-galactose	1.5	2.5
p-Nitrophenyl- $\beta$ -D-galactose	3.13	5.0
L-Fucose	NI	NI
1-O-Methyl- $\alpha$ -D-glucose	>200	NI
1-O-Methyl- $\alpha$ -D-mannose	>200	NI
Protein concentration	350 ng	60 ng

NI, No inhibition.

mixture of sodium metaperiodate and sodium cyanoborohydride at pH 3.5 resulted in the oxidative cleavage of the carbohydrates and the reduction of the aldehyde groups to primary alcohols. By conducting the procedure at acid pH, the possibility of Schiff's base formation between the aldehyde groups and amino groups in the protein and the non-specific oxidation of amino acids was minimised. The modified lectin had lost its haemagglutinating activity. The results of chemical modifications of amino acids and saccharide side chains are summarised in table 2.

Native unmodified lectin of our preparation did not bind to any of the immobilised lectins, concanavalin A, wheat germ agglutinin, and  $\beta$ -galactose-specific RCA1 nor to cross-linked guar gum. But the protein could be bound to  $\alpha$ -galactose-specific jack-fruit seed agglutinin (Jacalin) which binds specifically the immunoglobulin A (IgA) containing D-galactose residues (Roque-Barriera and Campos-Neto, 1985). It is difficult to explain the non-binding to RCA1, since the lectin binds to Jacalin as both these plant agglutinins recognise terminal D-galactose residues. This result does not necessarily suggest a terminal  $\alpha$ -linked galactose in the placenta lectin. IgA which contains only  $\beta$ -linked terminal galactose (Baenziger and Kornfeld, 1974) binds to Jacalin (Roque-Barriera and Campos-Neto, 1985).

Human placenta galactose-binding protein is different from human hepatic lectin. Hepatic lectin consists of a single subunit of 41 kDa glycoprotein and required detergent (Triton-X-100) for extraction from the tissue. It also required detergent and  $\text{Ca}^{2+}$  for binding to the ligand (Baenziger and Maynard, 1980). Placenta protein resembled more the galactose-binding lectin of bovine heart and brain (Carding *et al.*, 1985). One difference noted between bovine heart lectin and the present one is that in the latter we were unable to locate any high molecular weight fraction by gel

**Table 2.** Chemical modification of amino acid groups and oligosaccharide chains.

Modifying reagent	Amino acid group or oligosaccharide modified	Minimum concentration of protein (ng) required for agglutination			
		Trypsinised rabbit cells		Trypsinised human cells (B + ve)	
		Control*	Test	Control*	Test
None	—	60	60	350	350
Trinitrobenzene sulphonate	— NH <sub>2</sub>	830	NA	3300	NA
Citraconic anhydride	— NH <sub>2</sub>	560	NA	2240	NA
Maleic anhydride	— NH <sub>2</sub>	540	NA	2150	NA
Dithionitrobenzene	— SH	260	NA	1050	NA
p-hydroxymercuribenzoate	— SH	142	NA	1050	NA
N-Acetyl imidazole	Tyr	580	580	2300	2300
Diethyl pyrocarbonate	His	500	NA	2000	NA
Phenyl glyoxal hydrate	Arg	1700	NA	4300	NA
2-Hydroxy nitrobenzyl bromide	Trp	580	580	2300	2300
2-Hydroxy nitrobenzyl bromide	Trp	580	580	4600	4600
2-Methoxy nitrobenzyl bromide	Trp	580	580	4600	4600
Sodium metaperiodate, pH 4.5	Oligosaccharides	500	NA	2200	NA
Sodium metaperiodate (40 mM) and sodium cyanobromide (80 mM) pH 3.5	Oligosaccharides	600	NA	ND	ND

\*Identical conditions as required for test without the modifying reagent.

NA, No agglutination.

ND, Not done.

**Table 3.** Amino acid composition of human placenta galactose-binding protein.

Amino acid	Mol/mol of protein <sup>a</sup>
K	11.36
H	3.08
R	6.60
B	19.72
T	9.49
S	12.24
Z	20.60
P	9.07
G	18.31
A	20.86
C <sup>b</sup>	2.0
V	9.51
M	1.41
I	6.25
L	13.12
Y	2.51
F	12.85
W <sup>c</sup>	0.0

Amino acid residues are denoted by single letter.

<sup>a</sup>Minimum molecular weight 26,900.

<sup>b</sup>Cysteine was determined by DTNB method.

not be extracted with buffer solutions alone but required lactose. It may be presumed that the lectin remains attached to the oligosaccharide chains of the glycoconjugates on the membrane and lactose replaces the oligosaccharide chains during extraction process.

## Acknowledgement

This work was supported by a grant from Department of Science and Technology, New Delhi. The authors thank Mrs. Annama Kuriakose for excellent technical assistance. The mitogenic study was carried out at the laboratory of Dr. M. R. Das and Dr. Gopal Pande of Centre for Cellular and Molecular Biology, Hyderabad. We thank Mr. N. Sitaram of the same Institute for the amino acid analyses.

## References

- Andrews, R. (1965) *Biochem. J.*, **96**, 595.
- Appukuttan, P. S., Surolia, A. and Bachhawat, B. K. (1977) *Indian J. Biochem. Biophys.*, **14**, 382.
- Appukuttan, P. S. and Basu, D. (1981) *Anal. Biochem.*, **113**, 253.
- Ashwell, G. (1977) *Mammalian Cell Membranes*, **4**, 57.
- Ashwell, G. and Harford, J. (1982) *Annu. Rev. Biochem.*, **51**, 531.
- Atassi, M. Z. and Habeeb, A. F. S. A. (1972) *Methods Enzymol.*, **25**, 546.
- Axen, R., Porath, J. and Ernback, S. (1967) *Nature (London)*, **214**, 1302.
- Baenziger, J. U. and Kornfeld, S. (1974) *J. Biol. Chem.*, **249**, 7260, 7270.
- Baenziger, J. U. and Maynard, Y. (1980) *J. Biol. Chem.*, **255**, 4613.
- Barondes, S. H. (1984) *Science*, **223**, 1259.
- Bencze, W. L. and Schmid, K. (1957) *Anal. Chem.*, **29**, 1193.
- Bloch, R. and Burger, M. M. (1974) *Biochem. Biophys. Res. Commun.*, **58**, 13.
- Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248.
- Butler, P. J. G., Harris, J. I., Hartley, B. S. and Leberman, R. (1969) *Biochem. J.*, **112**, 679.
- Carding, S. R., Childs, R. A., Thorpe, R., Spitz and Feizi, T. (1985) *Biochem. J.*, **228**, 147.
- Davis, B. D. (1964) *Ann. N.Y. Acad. Sci.*, **121**, 404.
- deWaard, A., Hickman, S. and Kornfeld, S. (1976) *J. Biol. Chem.*, **251**, 7581.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1965) *Anal. Chem.*, **28**, 350.
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.*, **82**, 70.
- Fields, R. (1972) *Methods Enzymol.*, **25**, 464.
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.*, **25**, 457.
- Hirabayashi, T. and Kasai, K. I. (1984) *Biochem. Biophys. Res. Commun.*, **122**, 938.
- Laemmli, U. K. (1970) *Nature (London)*, **227**, 680.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Miles, E. W. (1977) *Methods Enzymol.*, **47**, 431.
- Riordian, J. F. and Vallee, B. L. (1972) *Methods Enzymol.*, **25**, 449, 500.
- Roque-Barreira, M. C. and Campos-Neto, A. (1985) *J. Immunol.*, **13**, 1740.
- Savage, W. E. and Fontana, A. (1977) *Methods Enzymol.*, **47**, 442.
- Smith, E. L. (1977) *Methods Enzymol.*, **47**, 156.
- Spiro, R. G. (1966) *Methods Enzymol.*, **8**, 26.
- Sureshkumar, G., Appukuttan, P. S. and Basu, D. (1982) *J. Biosci.*, **4**, 257.
- Surolia, A., Prakash, N., Bishayee, S. and Bachhawat, B. K. (1973) *Indian J. Biochem. Biophys.*, **10**, 145.
- Takahashi, K. (1968) *J. Biol. Chem.*, **243**, 6171.
- Thorpe, P. E., Detre, S. I., Foxwell, B. M. J., Brown, A. N. F., Skilleter, D. N., Wilson, G., Forrester, J. A. and Stripe, F. (1985) *Eur. J. Biochem.*, **147**, 197.

## Effect of chemical modification on structure and activity of glucoamylase from *Aspergillus candidus* and *Rhizopus* species

B. C. SHENOY\*, A. G. APPU RAO and  
M. R. RAGHAVENDRA RAO\*\*†

Central Food Technological Research Institute, Mysore 570 013, India

Present address: \*Biochemistry Department, Case-Western Reserve University Cleveland, Ohio 44106, USA

\*\*SREE, 409, 12th Main Road, Saraswathipuram, Mysore 570 009, India

**Abstract.** The histidine, tyrosine, tryptophan and carboxyl groups in the enzyme glucoamylase from *Aspergillus Candidus* and *Rhizopus* species were modified using group specific reagents. Treatment of the enzyme with diethylpyrocarbonate resulted in the modification of 0.3 and 1 histidine residues with only a slight loss in activity (10% and 35%) of glucoamylase from *Aspergillus candidus* and *Rhizopus* species respectively. Modification of tyrosine either by N-acetylimidazole or [I<sup>125</sup>]-leads to a partial loss of activity. Under denaturing conditions, maltose did not help in protecting the enzyme against tyrosine modification or inactivation. Treatment with 2-Hydroxy-5-nitro benzyl bromide in the presence of urea, photooxidation at pH 9.0, N-bromosuccinamide at pH 4.8 resulted in a complete loss of activity. However, the results of experiments in the presence of maltose and at pH 4.8 photooxidation and N-bromosuccinamide treatment suggested the presence of two tryptophan residues at the active site. There was a complete loss of enzyme activity when 10 and 28 carboxyl groups from *Aspergillus candidus* and *Rhizopus*, respectively were modified. Modification in the presence of substrate maltose, showed at least two carboxyl groups were present at the active site of enzyme and that only one active center seems to be involved in breaking all 3 types of  $\alpha$ -glucosidic linkages namely  $\alpha$ -1,4,  $\alpha$ -1,6 and  $\alpha$ -1,3.

**Keywords.** Glucoamylases; chemical modification; structure and activity.

## Introduction

Glucoamylase [ $\alpha$ -1,4-D glucan glucohydrolase EC 3.2.1.3] catalyses the hydrolysis of starch, producing D-glucose as the main product. Our studies on the structure and stability of glucoamylase II from *Aspergillus niger* suggested that the environment around aromatic amino acids is critical for activity and binding of the synthetic substrate, and that *p*-nitrophenyl- $\alpha$ -D-glucoside perturbed the environment around aromatic amino acids and caused a decrease in the ordered structure (Shenoy *et al.*, 1984). Glucoamylases from different fungal sources differ from one another in their physical and chemical properties (Manjunath *et al.*, 1983; Shenoy 1984). In the case of glucoamylase from *A. niger* and *A. saitoi* the presence of tryptophan and carboxyl groups at the active site is implicated (Jolley and Gray, 1976; Inokuchi *et al.*, 1982b; Ohnishi and Hiromi, 1976; Frankel-Conrat, 1957; Barker *et al.*, 1971; Gray and Jolley, 1973; Hoschke *et al.*, 1976). In addition, tyrosine and histidine residues have been identified at the active site (Zbereb'tosov *et al.*, 1976; Hoschke *et al.*, 1980a). Investi-

gation have been conducted to ascertain whether these amino acid residues are also present at the active site of the glucoamylases of *A. candidus* and *Rhizopus* species. The tryptophan, tyrosine, histidine and carboxyl groups in glucoamylase from *A. candidus* and *Rhizopus* species were modified with group specific reagents and the effect of such modification on activity and on the secondary and tertiary structure are reported in this communication.

## Materials and methods

Glucoamylase from *A. candidus* was a gift from Hindustan Antibiotics Ltd., Pimpri. *Rhizopus* glucoamylase, diethyl pyrocarbonate, N-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide (HNBB), N-bromosucciniamide (NBS), glycine methyl ester, p-chloromercuribenzoate, iodoacetamide, chloramine-T, N-ethylmaleimide, diisopropyl-fluorophosphate, bovine serum albumin (BSA), maltose, isomaltose and nigerose were from the Sigma Chemical Company, St. Louis, Missouri, USA. Urea and 1-ethyl-3 (dimethylaminopropyl) carbodiimide (EDC) were from Pierce Chemical Company, Rockford, Illinois, USA. Bolton and Hunter reagent [N-succinimidyl-3-(4-hydroxyl-5 [ $^{125}$ I] iodophenyl) propionate] and [ $^{125}$ I] were Radiochemical Centre, Amersham, England. All other chemicals used were of analytical reagent grade.

Glucoamylase from *A. candidus* and *Rhizopus* species were from purified (Shenoy, 1984) and activity determined using soluble starch as substrate (Manjunath and Raghavendra Rao, 1979). One unit of enzyme activity is defined as that of enzyme which produces one  $\mu$ mol of glucose per min under standard conditions. Specific activity is expressed as units per mg protein. The glucoamylase from *A. candidus* has a molecular weight ( $M_r$ ) of 71,000 and specific activity of 436 units\*.

Modification of histidyl residues were carried out according to the method of Kumagai *et al.* (1975) using diethyl pyrocarbonate.

Tyrosyl residues were modified according to Riordon *et al.* (1965) by using 400 fold molar excess of N-acetylimidazole. Iodination of the enzyme was carried out using the chloramine-T method (Greenwood and Hunter, 1963). The reaction mixture contained 0.01 ml of enzyme (0.5 mg/ml) 0.01 ml of 0.05 M phosphate buffer, pH 7.5, 0.02 ml of [ $^{125}$ I] (1 mCi/20  $\mu$ l), 0.01 ml of chloramine-T (4 mg/ml). The reaction was carried out for 10 s and stopped with the addition of 0.05 ml of metabisulphate (4 mg/ml). Then, 0.01 ml of KI (10 mg/ml) was added and the mixture was loaded on a column of sephadex G-10 (0.9  $\times$  30 cm) and eluted with 0.01 M phosphate buffer pH 7.5 containing 0.15 M saline and 0.5% albumin. The extent of iodination of the protein was calculated from the radioactivity incorporated and the quantity of the enzyme protein and the known specific activity of the iodine. Iodination was also carried out by using iodinated ester of N-succinimidyl 3-(4 hydroxy 5 [ $^{125}$ I]-iodophenyl) propionate according to Bolton and Hunter (1973). Three methods were used for the modification of tryptophan residues. Photooxidation was carried out by the method Barker *et al.* (1971) using 0.001% rose bengal and the tryptophan content of the enzyme was determined by the method of Spies and Chambers (1949).

method of Challa and Pihm (1976).

Modification of carboxyl groups was carried out according to the method of Gray and Jolley (1973). For amino acid analysis 500  $\mu$ l aliquots were withdrawn from the reaction mixture and chromatographed on Biogel P-10 column (0.9  $\times$  60 cm) equilibrated with 0.2 M acetate buffer pH 4.8. Fractions containing protein were pooled, hydrolysed and amino acid content determined (Shenoy, 1984).

Circular dichroism (CD) measurements were made with a JASCO-J20C automatic recording spectropolarimeter calibrated with d-10-camphor sulphonic acid. Quartz cells of different path length (1 cm, 0.1 cm) were used for measurements in the region 350–200 nm. Slits were programmed to yield a band width of 10 Å at each wavelength. Mean residue ellipticities  $[\theta]_{\text{mrw}}$  were calculated by standard procedures (Adler *et al.*, 1973). A value of 110 for mean residue weight was used. The CD spectra were analysed by the method of Provencher and Glockner (1981) to estimate the secondary structure.

## Results

### *Modification of histidine residues*

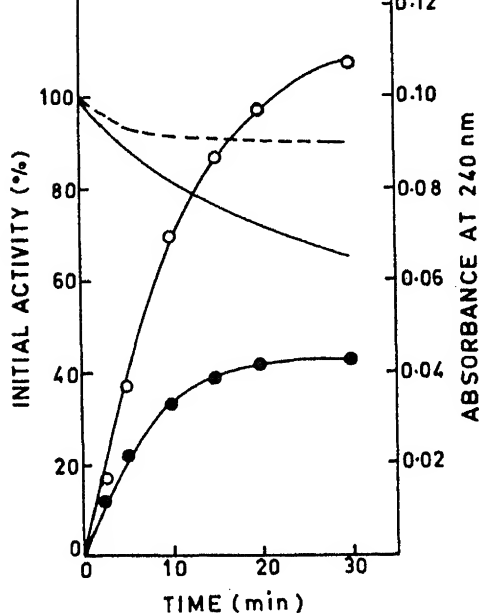
The results of the study of the effect of the treatment with diethyl pyrocarbonate on activity and histidine content of glucoamylases are shown in figure 1. By the procedure adopted only 0.3 and 1 residue could be modified in *A. candidus* and *Rhizopus* glucoamylases, respectively. With the modification of 0.3 residue of histidine around 10% of activity was lost in the case of *A. candidus* glucoamylase where as in the case of *Rhizopus* species around 35% of activity was lost with the modification of one residue in 30 min.

### *Modification of tyrosine residues*

*N. acetylimidazole*: Acetylation of tyrosine groups in glucoamylase from the two sources was carried out both under non-denaturing conditions and in 8 M urea. In the absence of urea, only one tyrosine residue was modified in case of the *A. candidus* enzyme resulting in a 10% loss of activity. Although 4 tyrosine residues were modified in case of *Rhizopus* glucoamylase, activity loss was only 30%. In the presence of an unfolding reagent like 8 M urea, 5 and 9 tyrosine residues were modified by the reagent in the case of *A. candidus* and *Rhizopus* glucoamylases resulting in a 86% loss of activity in both cases. Substrate maltose did not protect the enzyme against modification by the reagent.

*Iodination of enzyme with radioactive I<sup>125</sup>*: Iodination of the glucoamylase from two species led to an incorporation of about 40% and 32% radioactive iodine in case of the enzymes *A. candidus* and *Rhizopus* species, respectively. The iodinated *A. candidus* enzyme retained only 32% of its original activity whereas iodinated enzyme from *Rhizopus* species retained 72% of its activity. Iodination with Bolton and Hunter





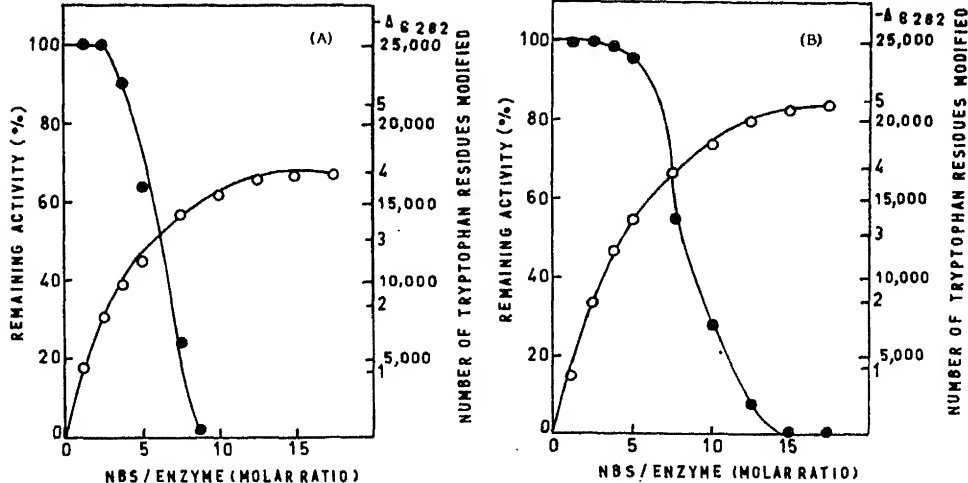
**Figure 1.** Effect of diethylpyrocarbonate on the enzymic activity and the histidine content of glucoamylases [2.5 ml of enzyme solution (3 mg) in 0.01 M potassium phosphate buffer pH 6.0, treated with freshly prepared 0.05 ml of diethyl pyrocarbonate (0.08 M in cold ethanol at 6°C for 30 min (control enzyme treated with cold ethanol)  $E_M = 3200\text{M}^{-1}\text{cm}^{-1}$  for carboethoxyl histidyl residues in protein at 242 nm].

(—), Activity of *A. candidus* glucoamylase; (---), activity of *Rhizopus* glucoamylase; (●), Absorbance at 240 nm of *A. candidus* glucoamylase; (○), Absorbance at 240 nm *Rhizopus* glucoamylase.

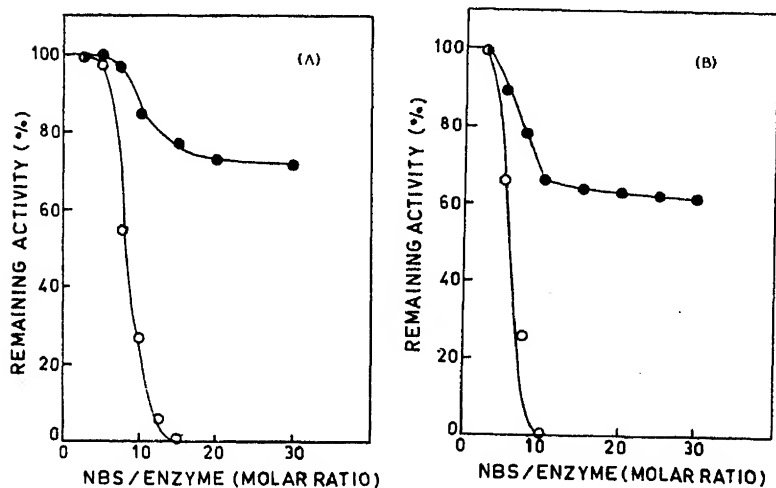
reagent was much less effective and resulted in the incorporation of about 4% of radioactivity and retention of 90~100% of activity.

### Modification with NBS

Modification of tryptophan residues with NBS at different pH values resulted in modification to different extents with varying residual activity. At pH 4.8, the pH of optimum activity, treatment of the enzyme with NBS led to the modification of 5 and 4 tryptophan residues from *A. candidus* and *Rhizopus* species respectively and to total loss of activity (figure 2). At this pH, and in the presence of substrate, maltose, only 3 and two tryptophan residues were modified in the glucoamylases of *A. candidus*, and *Rhizopus* respectively and the modified enzymes had 60–65% activity (figure 3). At pH 6.0 only two tryptophan residues were modified by NBS in enzymes from both the species and the modified enzymes of *A. candidus* and *Rhizopus* retained 75 and 65% of its activity, respectively. At pH 7.0 NBS modified only 0.3 and 0.2 residues of tryptophan of the two enzymes and had no effect on the activity.



**Figure 2.** The effect of chemical modification of glucoamylase by NBS at pH 4.8: Enzyme;  $8 \mu\text{M}$ ,  $0.05 \text{ M}$  acetate buffer pH 4.8,  $25^\circ\text{C}$  (the number of tryptophan residues modified by NBS was calculated from decrease in the molar difference absorbance coefficient at  $282 \text{ nm}$ ,  $\epsilon_M = 4200$ ). (○), Decrease in molar difference absorption coefficient at  $282 \text{ nm}$ ; (●), the remaining activity of the enzyme. (A) *A. candidus*; (B) *Rhizopus* species.



**Figure 3.** The effect of substrate on the chemical modification by NBS. Chemical modification: Enzyme,  $8 \mu\text{M}$ ,  $0.05 \text{ M}$  acetate buffer, pH 4.8,  $25^\circ\text{C}$ .

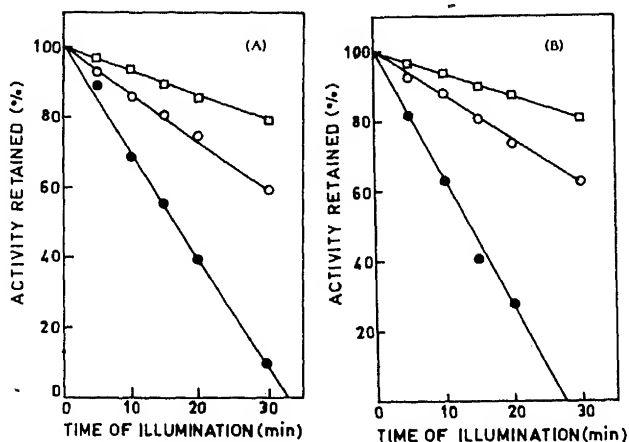
The remaining activity of the enzyme modified in the absence of substrate maltodextrin (○) and the enzyme modified in the presence of substrate ( $0.19\%$ ) (●) are shown.

(A) *A. candidus*; (B) *Rhizopus* species.

### Modification with HNBB

Under non denaturing conditions, 3 and 2 tryptophan residues of the glucoamylase

The results of photooxidation are shown in figure 4. After photooxidation for 30 min at pH 4.8, the enzyme from the two species had 63 and 60% activity with the modification of 3 and 2 tryptophan residues, respectively. In the presence of maltose, the substrate, there was only a 20% loss of activity in enzyme from both the species and only one tryptophan residue was modified. Photooxidation of the enzymes at pH 9.0 resulted in a complete loss of activity within 35 min from both the species. Eight tryptophan residues were modified in case of *A. candidus* and 4 tryptophan residues were modified in case of *Rhizopus* enzyme. Amino acid analysis of the enzyme and also the estimation of tryptophan content of the enzyme showed that only tryptophan groups, and neither histidine nor tyrosine residues were modified by photooxidation (Shenoy, 1984).



**Figure 4.** The photooxidation of glucoamylases in the presence of rose Bengal at pH 4.8 (○); pH 9.0 (●), and in the presence of maltose (0–15 M) at pH 4.8 (□). Solution at each pH value contained glucoamylase (1 mg) and rose Bengal (10 µg) in an initial volume of 1.1 ml. Maltose (30 mg) was added to 0.6 ml of enzyme die solution immediately prior to photooxidation. The temperature of reaction was 4°C. (A) *A. candidus*; (B) *Rhizopus* species.

### UV-difference spectra

To gain a better understanding of the role of aromatic amino acids in glucoamylase, difference-spectral measurements were made in the presence of substrate maltose. The difference spectra (figure 5) in the presence of substrate maltose, are seen with troughs at 281, 289 and 300 nm in case of the *candidus* enzyme and 278, 289 and 302 nm with *Rhizopus* enzyme. The trough near 300 and 302 nm disappeared with

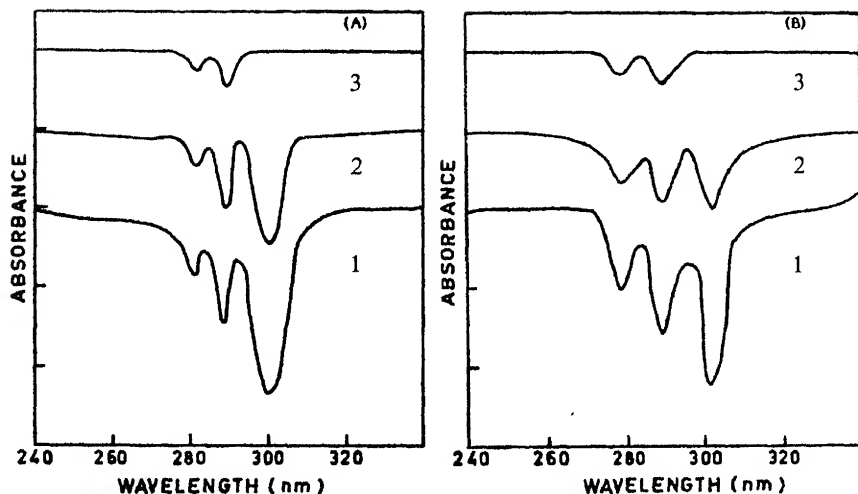


Figure 5. A. Difference spectra produced by maltose on the native and NBS-modified *A. candidus* glucoamylase. Enzyme, 30  $\mu$ M; NBS (1) 0 M (native enzyme) (2) 0.27 mM (NBS/enzyme = 8.9). (3) 0.45 mM (NBS/enzyme = 15.0), 0.05 M acetate buffer, pH 4.8, 25°C.

The difference spectra were measured at pH 4.8 and 25°C with 10  $\mu$ M glucoamylase and 97 mM maltose.

B. Difference spectra produced by maltose of the intact and NBS-modified *Rhizopus* glucoamylase.

Enzyme 30  $\mu$ M, NBS (1) 0 M (native enzyme); (2) 0.18 mM (NBS/enzyme = 6.0) and (3) 0.34 mM (NBS/enzyme = 11.3), 0.05 M acetate buffer, pH 4.8 25°C.

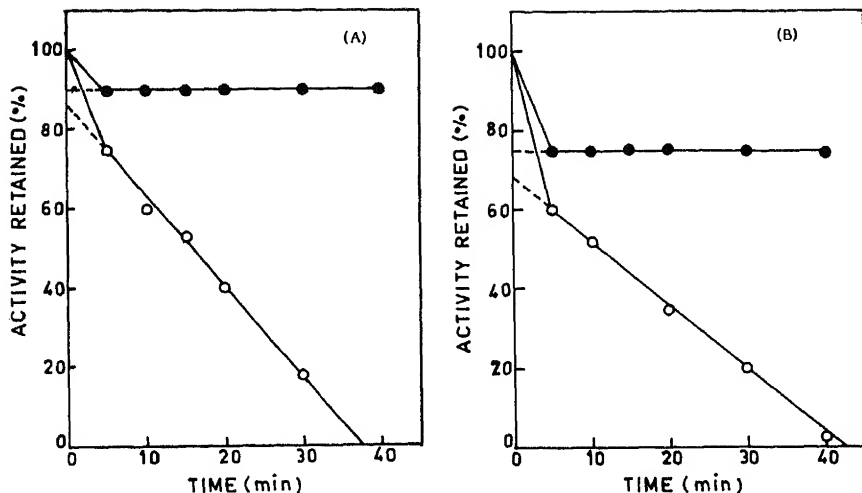
The difference spectra were measured at pH 4.8 and 25°C with 10  $\mu$ M glucoamylase and 97 mM maltose.

the initiation of modification of the tryptophan groups with NBS followed by a loss in enzyme activity.

Modification of carboxyl groups resulted in a complete loss of activity with in 40 min in both *A. candidus* (figure 6) and *Rhizopus* glucoamylases. However, in the presence of substrate maltose, only 10 and 25% activities were lost in the glucoamylase of *A. candidus* and *Rhizopus* species respectively. In the absence of substrate 10 glycine residues were incorporated into a mol of glucoamylase from *A. candidus* and in the presence of substrate 8 residues were incorporated. 28 and 26 glycine residues were incorporated per mol of glucoamylase from *Rhizopus* species, in the absence and in presence of substrate respectively.

#### Modification by other group specific reagents

In addition to the above reagents, different reagents specific for free sulphhydryls, serine hydroxyls and divalent metal ions were added separately to the enzyme solution to the requisite concentration. After incubation for 30 min at 6–8°C, the enzyme activity was assayed at 60°C. In enzyme from both the species, 20 mM EDTA, 2 mM *p*-chloromercuribenzoate, 2 mM Iodoacetamide, 2 mM N-ethyl



**Figure 6.** A. Loss of activity of glucoamylase from *A. candidus* on treatment with glycine methyl ester and 1-ethyl-1 (3-dimethylaminopropyl) carbodiimide in absence (○) and in the presence of (●) maltose. The intercepts shown are calculated to be; maltose absent, 86%; maltose present, 90%.

**B.** Loss of activity of glucoamylase from *Rhizopus* species with glycine methyl ester and 1-ethyl-1-(3-dimethylaminopropyl) carbodiimide in absence (○) and in presence of maltose (●). The intercepts shown are calculated to be: maltose absent, 68%; maltose present, 75%.

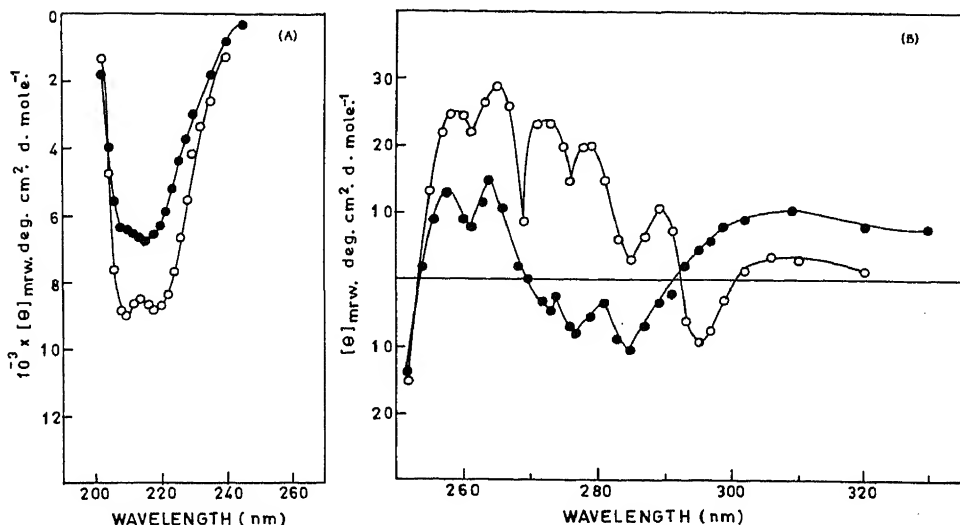
In order to know whether there were more than one active center for breaking of 3 types of bonds namely  $\alpha$ -1,4,  $\alpha$ -1,6 and  $\alpha$ -1,3, the carboxyl-modified enzyme in the presence of substrate maltose was tested against two other types of substrate, *e.g.* isomaltose and nigerose. Both the substrate were hydrolysed by the enzyme thus showing that the enzyme contains only one active center for the breakdown of all the 3 types of substrates.

#### *Effect of tyrosine modification on structure*

The modification of tyrosine residues in the absence of denaturing agent resulted in modification of one tyrosine residue in case of glucoamylase from *A. candidus*. Modification of this residue did not affect near UV CD bands, (except for a slight diminution in the 295 nm band) nor it affected the secondary structure of enzyme.

#### *Effect of Carboxyl groups modification on structure*

Modification of carboxyl groups in the absence of substrate resulted in changes in the near UV and far UV CD bands. The CD spectra of the enzyme from *A. candidus* in 0.05 M acetate buffer at pH 4.8 before and after carboxyl group modification is shown in figure 7. In the near UV region, the enzyme exhibited peaks at 304–305, 289, 279, 272, 265 and 257 nm and a trough at 295 nm. After modification, the peaks



**Figure 7.** Near UV and far UV CD spectra of glucoamylase from *A. candidus* before and after carboxyl group modification pH 4.8. 0.05 M. Acetate buffer (○) control (●) carboxyl groups modified.

suggest a change in the tertiary structure of enzyme resulting an altered conformation around aromatic aminoacids. In the far UV region, there is a decrease in the ordered structure of enzyme. The analysis of secondary structure of enzyme according to the method of Provencher and Glockner (1981) suggest that the native enzyme has 17%  $\alpha$ -helix, 38%  $\beta$ -structure and 45% aperiodic structure. After modification the helical content decreased to 8%,  $\beta$ -structure 29% and aperiodic structure increases to 63%. Thus due to carboxyl group modification there was a change in the secondary and tertiary structure of enzyme.

## Discussion

The glucoamylase from *A. candidus* species contains 4 histidine residues (table 1). Only a fraction of histidine residues were available for the reagent diethyl pyrocarbonate and the modified enzyme had 90% of its original activity. This implies that histidine groups are not easily accessible for reagent and are not part of active site of the enzyme. The non accessibility of histidine residues could be due to the presence of carbohydrate moiety. Of the total 3 histidine residues of *Rhizopus* enzyme only one residue is modified by the reagent with a 35% loss in activity. Hoschke *et al.* (1980) and Zherebtosov *et al.* (1976) have shown in the case of glucoamylase from *A. niger* modification of histidine residues decreases the binding affinity of the substrate to enzyme and are located away from the catalytic site. Thus it is probable histidine residues are only involved in binding of substrate and are not part of catalytic site.

Glucoamylases from *A. candidus* and *Rhizopus* species contain total of 22 and 23 tyrosine residues, respectively (table 1). In the absence of urea, N-acetyl imidazole

Reagent	<i>A. candidus</i>		<i>Rhizopus species</i>	
	Activity	No. of residues modified	Activity	No. of residues modified
<i>Histidine groups modification</i>		4		3
Diethyl pyrocarbonate	90	0.3	65	1
<i>Tyrosine groups modification</i>		22		23
1. N-acetylimidazole				
(a) in absence of urea	85	1	70	4
(b) in presence of urea	14	5	15	9
(c) in presence of urea + substrate	16	5	21	9
2. Iodine 125 ( <sup>125</sup> I)	32 (40)	—	72 (32)	—
<i>Tryptophan groups modification</i>		13		8
1. 2-Hydroxy-5-nitro benzyl bromide				
(a) in absence of urea	60	3	65	2
(b) in presence of urea	0	12	0	6
2. Photooxidation (rose bengal)				
(a) pH 4.8	63	3	60	2
(b) pH 4.8 + substrate	80	1	80	1
(c) pH 9.0	0	8	0	4
3. N-bromosuccinimide				
(a) pH 4.8	0	5	0	4
(b) pH 4.8 + substrate	62	3	66	2
(c) pH 6.0	75	2	65	2
(d) pH 7.0	100	0.3	100	0.2
4. Carboxyl groups modification		46 + 28*		55 + 22*
1. Glycine methyl ester and 1-ethyl-1(3-dimethyl amino-propyl) carbodiimide				
(a) in absence of substrate	0	10	0	28
(b) in presence of substrate	90	8	75	26

\*Total number of Asp and Glu residues.

residue of glucoamylase from *A. candidus* in the absence of denaturing agent by N-acetylimidazole did not affect its secondary and tertiary structure. Exposed tyrosine residues are modified by this reagent (Riordan and Valle, 1972) and with the unfolding of enzyme in 8 M urea, all the tyrosine residues are modified with complete loss in activity of the enzyme. It is probable that tyrosine residues which are not exposed to solvent are at the active center and the part of the active center may be in the hydrophobic interior of the molecule. Substrate maltose did not protect the enzyme against modification/inactivation. From our CD measurements in the presence of synthetic substrate *p*-nitrophenyl- $\alpha$ -D-glucoside, in the case of *A. niger* glucoamylase, the intensity of the 277 nm band in near UV CD spectra decreases with increasing concentration of substrate suggesting the possibility of tyrosine being a part of the binding site. With modification, binding ability of substrate might be lost with the resultant decrease in activity. Hoschke *et al.* (1980a,b), have identified tyrosine groups at the active site of the enzyme and showed that binding affinity of the substrate decreased by the modification of tyrosine without any appreciable decrease in catalytic activity. Earlier we had suggested (Shenoy *et al.*, 1984) that the

activity of the enzyme is very sensitive to the environment around aromatic amino-acids in glucoamylases.

Difference spectra measurements conducted in the presence of substrate both before and after modification of tryptophan residues suggest that tryptophan residues are involved in binding of substrate and that due to binding of the substrate the environment around tryptophan changes. The blue shift in the spectrum suggests the transfer of tryptophan groups from a hydrophobic region of high refractive index to the solvent environment of lower polarisability although rupture of hydrogen bonds cannot be excluded.

Tryptophan residues from the enzyme are modified to different extent with change in pH. At pH 4.8, the optimum pH of the enzyme, NBS modified 5 tryptophan residues from *A. candidus* and 4 tryptophan residues from *Rhizopus* species with total loss in activity. Lowering the pH from 7.0-4.8, results in a change in conformation of enzyme (Shenoy, B. C., Appu Rao A. G. and Raghavendra Rao, unpublished results) and also the availability of tryptophan residues to NBS. At pH 7.0 enzyme has no activity and only a fraction of tryptophan groups are modified by the reagent. These results suggest a direct involvement of tryptophan groups in catalytic activity. In the presence of substrate maltose at pH 4.8 only 3 and 2 tryptophan residues from *A. candidus* and *Rhizopus* species enzyme respectively were modified, while 62 and 66% of activity remained suggesting the protection of tryptophan residues by the substrate. There is a difference in the reactivity of tryptophan residues towards the HNBB reagent under denaturing and non denaturing conditions. Modification under denaturing conditions results in an enzyme with a complete loss of activity. Photooxidation of tryptophan residues suggest the involvement of these residues in catalytic activity.

Carboxyl groups on the enzyme seem to play an important role in catalytic activity. Modification of 10 and 28 of these groups from *A. candidus* and *Rhizopus* enzyme results in a complete loss of activity. In the presence of substrate, two carboxyl groups less are modified in both the enzymes which has retained most of its activity suggesting that at least two carboxyl groups are at the active site. These results are in conformity with the results of Jolley and Gray (1976) and Hiromi *et al.* (1966) have suggested that active form of carboxyl groups at the active site are  $\text{COO}^-$  and  $\text{COOH}$  of the enzyme.

Modification of carboxyl groups resulted in a change of secondary and tertiary structure of glucoamylase from *A. candidus*. In case of glucoamylase from *A. saitoi* modification of carboxyl groups with  $[^{14}\text{C}]$ -1-cyclohexyl-3 (2-morpholinyl (4)-ethyl) carbodimide resulted in complete loss of activity without change in secondary and tertiary structure of enzyme (Inokuchi *et al.*, 1982).

Only one active center seems to be involved in breaking all the 3 types of  $\alpha$ -glucosidic linkages namely  $\alpha$ -1,4,  $\alpha$ -1,6 and  $\alpha$ -1,3. An enzyme preparation whose carboxyl groups were modified in the presence of maltose hydrolysed both isomaltose and nigerose completely thus indicating that the glucoamylase contained only one active site for breaking the glucosidic bonds in maltose, isomaltose and nigerose.



- Bolton, A. E. and Hunter, W. M. (1973) *Biochem. J.*, **133**, 529.
- Frankel-Conrat, H. (1957) *Methods Enzymol.*, **4**, 247.
- Gray, C. J. and Jolley, M. E. (1973) *FEBS Lett.*, **29**, 197.
- Greenwood, F. E. and Hunter, W. M. (1963) *Biochem. J.*, **89**, 114.
- Hiromi, K., Takahashi, K., Hamauza, Z. and Ono, S. (1966) *J. Biochem. (Tokyo)*, **59**, 469.
- Hoschke, A., Laszlo, E. and Hollo, J. (1976) *Diesterke*, **28**, 426.
- Hoschke, A., Laszlo, E. and Hollo, J. (1980a) *Carbohydr. Res.*, **81**, 145.
- Hoschke, A., Laszlo, E. and Hollo, J. (1980b) *Carbohydr. Res.*, **81**, 157.
- Inokuchi, N., Takahashi, T. and Yashimoto, A. (1982b) *J. Biochem. (Tokyo)*, **91**, 1661.
- Inokuchi, N., Iwasawa, M., Takahashi, T. and Irie, M. (1982a) *J. Biochem. (Tokyo)*, **91**, 125.
- Jolley, M. E. and Gray, C. J. (1976) *Carbohydr. Res.*, **49**, 361.
- Kumagai, H., Utagawa, T. and Yamada, H. (1975) *J. Biol. Chem.*, **250**, 1661.
- Manjunath, P. and Raghavendra Rao, M. R. (1979) *J. Biosci.*, **1**, 409.
- Munjunath, P., Shenoy, B. C. and Raghavendra Rao, M. R. (1983) *J. Appl. Biochem.*, **5**, 235.
- Ohnishi, M. and Hiromi, K. (1976) *J. Biochem. (Tokyo)*, **79**, 11.
- Provencher, S. W. and Glockner, J. (1981) *Biochemistry*, **20**, 33.
- Riordan, J. F. and Vallee, B. L. (1972) *Methods Enzymol.*, **25**, 500.
- Riordan, J. F., Wacker, W. E. C. and Vallee, B. L. (1965) *Biochemistry (USA)*, **4**, 1758.
- Shenoy, B. C., Appu Rao, A. G. and Raghavendra Rao, M. R. (1984) *J. Biosci.*, **6**, 601.
- Shenoy, B. C. (1984) *Studies on amylases with special reference to microbial glucoamylases* Ph.D. thesis, University of Mysore, Mysore.
- Spies, J. R. and Chambers, D. C. (1949) *Anal. Chem.*, **21**, 1249.
- Terao, T. and Ukita, T. (1969) *Biochim. Biophys. Acta.*, **181**, 347.
- Venkataramu, K., Manjunath, P. and Raghavendra Rao, M. R. (1975) *Indian J. Biochem. Biophys.*, **12**, 107.
- Zherebtosov, N. A., Zabilina, L. F. and Ektova, A. I. (1976) *Biochemistry (USSR)*, **41**, 1717.

# Effect of alkaline pH on sunflower 11S protein

G. SRIPAD\* and M. S. NARASINGA RAO†

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570 013, India.

\*Present address: Department of Biochemistry, Kansas University Medical Centre, 9th and Rainbow Blvd., Kansas City, Kansas 66103, USA

**Abstract.** The effect of alkaline pH on sunflower 11S protein has been monitored by the techniques of ultracentrifugation, polyacrylamide gel electrophoresis, turbidity, viscosity, ultraviolet absorption spectra and fluorescence spectra. Both ultracentrifugation and polyacrylamide gel electrophoresis show the dissociation of the protein with increase in pH. Turbidity values decrease with pH while viscosity increases. With increase in pH, absorbance of the protein solution increases and there is a red shift in the absorption maximum. Fluorescence quenching and a red shift in the emission maximum are also observed. Both dissociation and denaturation of the protein occur. Analysis of turbidity, viscosity and fluorescence data suggests that apparently denaturation follows dissociation.

**Keywords.** Sunflower 11S protein; alkali; dissociation; denaturation.

## Introduction

Sunflower 11S protein binds chlorogenic acid below its isoelectric point (pH 5.0); it binds very little at neutral pH (Sastri, 1984). The protein is dissociated at pH 4.0 (Schwenke *et al.*, 1975). A study of the effect of low pH on 11S protein, monitored by a variety of techniques, shows that the protein undergoes dissociation and denaturation upto ~pH 3.0 and below this pH aggregation and renaturation occur. Therefore, it was of interest to study the effect of alkaline pH on the protein and compare it with that in acid pH.

## Materials and methods

### Materials

Sunflower seed (EC 68415) a Russian variety, grown in the State of Karnataka during the season, 1979–80, was obtained from Agro-Seed Corporation, Mysore. It was stored in the cold (~4°C). The sources of the chemicals used were: Sepharose 6B-100 from Sigma Chemicals Co., St. Louis, Missouri, USA; acrylamide, bisacrylamide from Koch-Light Laboratories, England; TEMED and  $\beta$ -mercaptoethanol from Fluka, A. G. Switzerland and ammonium persulphate and amido black from E. Merck, Germany.

Sunflower 11S protein was isolated by the method of Rahma and Narasinga Rao (1981) and further purified by gel filtration on Sepharose 6B-100. It was found to be homogeneous by gel electrophoresis and ultracentrifugation (Sripad and Narasinga Rao, 1987).

The measurements (except electrophoresis) were performed in water whose pH had been adjusted to the desired value by the addition of alkali.

*Ultracentrifugation:* The experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with rotor temperature indicator control unit and phase plate schlieren optics. A 1% protein solution was centrifuged at 60,000 rpm. Photographs were taken at different intervals of time and  $S_{20,w}$  was calculated (Schachman, 1959).

*Polyacrylamide gel electrophoresis (PAGE):* This was done in the range pH 9.0–10.0 using 0.005 M glycine-NaOH buffers. Gels (8%) were used and electrophoresis was performed at a constant current of 3 mA/tube. About 90  $\mu$ g of protein was loaded and bromophenol blue was used as indicator dye. The gels were stained for 30 min with 0.5% amido black in 7% acetic acid and destained by diffusion with 7% acetic acid.

*Viscosity:* The measurements were made at  $30^\circ \pm 0.1^\circ\text{C}$  using an Ostwald viscometer having a flow time of 173 s with distilled water. Protein (0.5%) solution adjusted to the desired pH value was equilibrated at  $30^\circ\text{C}$  for 30 min and the flow time recorded. Reduced viscosity was calculated from the flow time with the equation:

$$\eta_{\text{red}} = \frac{(t - t_0)}{t_0 C},$$

where  $t$  is the flow time with the solution;  $t_0$ , of the solvent and  $C$ , the protein concentration in g/ml.

*Turbidity:* It was obtained by absorbance measurements at 540 nm. Protein solution (0.18%) was used and the solution at pH 7.0 was used as the reference. Percent turbidity was calculated with the equation:

$$\% \text{ Turbidity} = \frac{T_0 - T}{T_0} \times 100,$$

where  $T_0$  is the transmittance of the protein solution at pH 7.0 and  $T$  of the protein at different pH values.

*Absorption spectrum:* It was recorded in Perkin-Elmer Model 124 recording spectrophotometer in the range 240–330 nm.

Model 205 spectrophotometer using 0.009% protein solution. The emission intensity was measured in the range 300–400 nm at room temperature ( $\sim 28^\circ\text{C}$ ) after excitation at 280 nm.

## Results and discussion

### *Ultracentrifugation*

The sedimentation velocity pattern of the 11S protein at pH 8.3 consisted of a single symmetrical peak with  $S_{20,w}$  value of 11.0 (figure 1). At pH 9.5, it consisted of 3 peaks with  $S_{20,w}$  values of 2.0, 7.0 and 11.0. At pH 10.5 also it consisted of 3 peaks. But the proportion of the 7S protein had increased while that of the 11S protein decreased. There was no change in the proportion of the 2S protein. At pH 11.5, the pattern consisted of only two peaks with  $S_{20,w}$  values of 0.7 and 3.5. Thus with increase in pH, dissociation of the protein to low molecular weight proteins occurred.

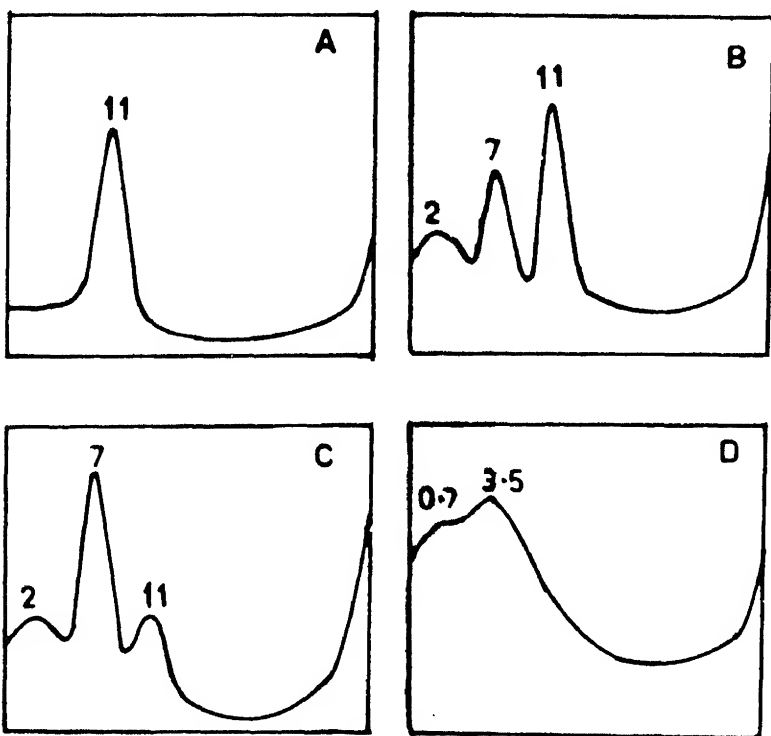


Figure 1. Effect of pH on the sedimentation velocity pattern of sunflower 11S protein (A), pH 8.3; (B), pH 9.5; (C), pH 10.5; (D), pH 11.5 (numbers indicate  $S_{20,w}$  values).

Kelley and Pressy (1966) have reported that at alkaline pH glycinin (of soybean) dissociates and Prakash and Nandi (1977) have reported that  $\alpha$ -globulin of sesame

### Electrophoresis

Electrophoresis was performed in the pH range 9.0 and 12.6 and the PAGE patterns are given in figure 2. At pH 8.3 and 9.0, the 11S protein gave a single band with low mobility and no other bands were observed. At pH 9.8 a faint fast moving band was observed in addition to the original band. The intensity of this band increased at pH 10.6. At pH 11.2, its intensity increased further with a concomitant decrease in that of the original band; in addition a faster moving band was also observed. The diffuse nature of the band suggested that it was polydisperse in nature. At pH 11.8, the band due to 11S protein almost disappeared with a decrease in the intensity of the second band also. There was an increase in the intensity of the diffuse fast moving band. At pH 12.6, only the diffuse, fast moving band was observed. Taken in conjunction with the sedimentation velocity data, PAGE experiments also indicated the dissociation of the 11S protein at higher pH values.

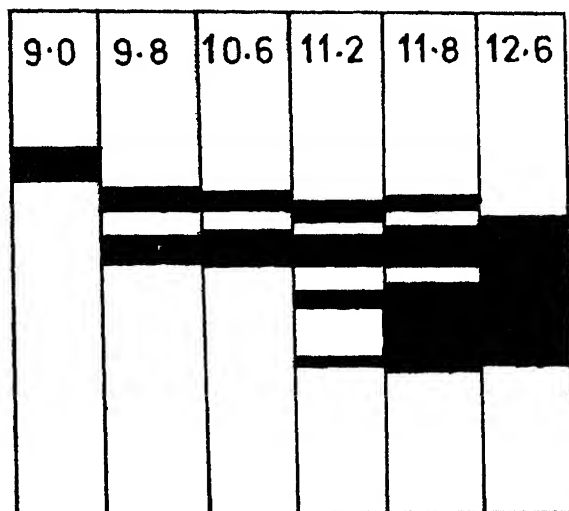


Figure 2. Effect of pH on the electrophoretic pattern of sunflower 11S protein (0.005 M glycine-NaOH buffers). Numbers refer to pH of measurement.

### Viscosity

The effect of alkaline pH on the reduced viscosity ( $n_{red}$ ) is shown in figure 3. Upto pH 10.2,  $n_{red}$  remained constant at the value of 4.2 ml/g. As the pH increased above pH 10.2,  $n_{red}$  increased and again a constant value of 10 ml/g was observed in the pH range 12.0–12.5. The midpoint of transition in the curve was around pH 11.5. The increase in  $n_{red}$  value indicated unfolding/expansion of the protein molecule (Joly,

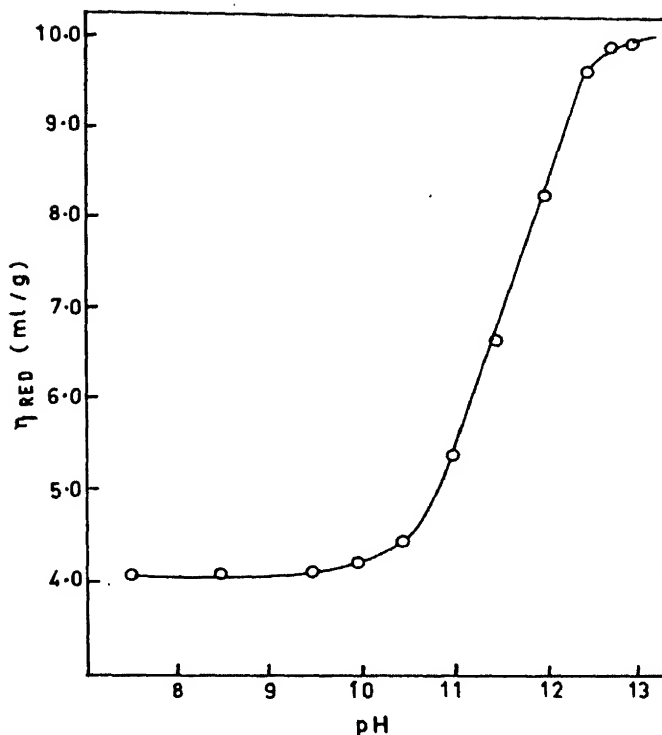


Figure 3. Effect of pH on the reduced viscosity of sunflower 11S protein.

### *Turbidity*

There was no change in the turbidity of the protein solution in the pH range 7.0–8.5 (figure 4). It decreased sharply upto pH 10.0 and then decreased gradually upto pH 12.8. Eventhough there was no dissociation in the region pH 8.5–9.0 the turbidity decreased. The decrease in turbidity above pH 9.0 could be due to the dissociation of the protein.

### *Absorption spectra*

The spectra recorded in the range 330–240 nm of the protein solution at different pH values showed considerable difference (figure 5). The spectrum in the pH range 7.4–11.0 consisted of a single maximum at 280 nm. A minimum in 250–270 nm region was also observed. However, as the pH increased the absorbance at the minimum increased and it showed a red shift also. Above pH 11.3 two maxima, one at 280 nm and the other at 288 nm, were observed. Evidently, the second maximum was due to the ionized tyrosine groups (Donovan, 1973). Upon ionization of phenolic group, the absorbance in the 280–290 nm region increases and the maximum shows a red shift. Thus the change in the shape of the protein spectrum at alkaline pH could be due to

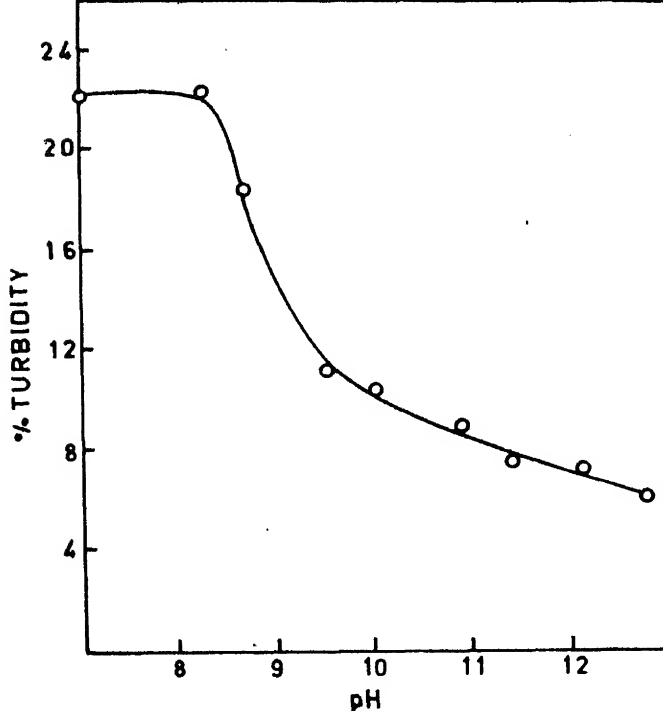
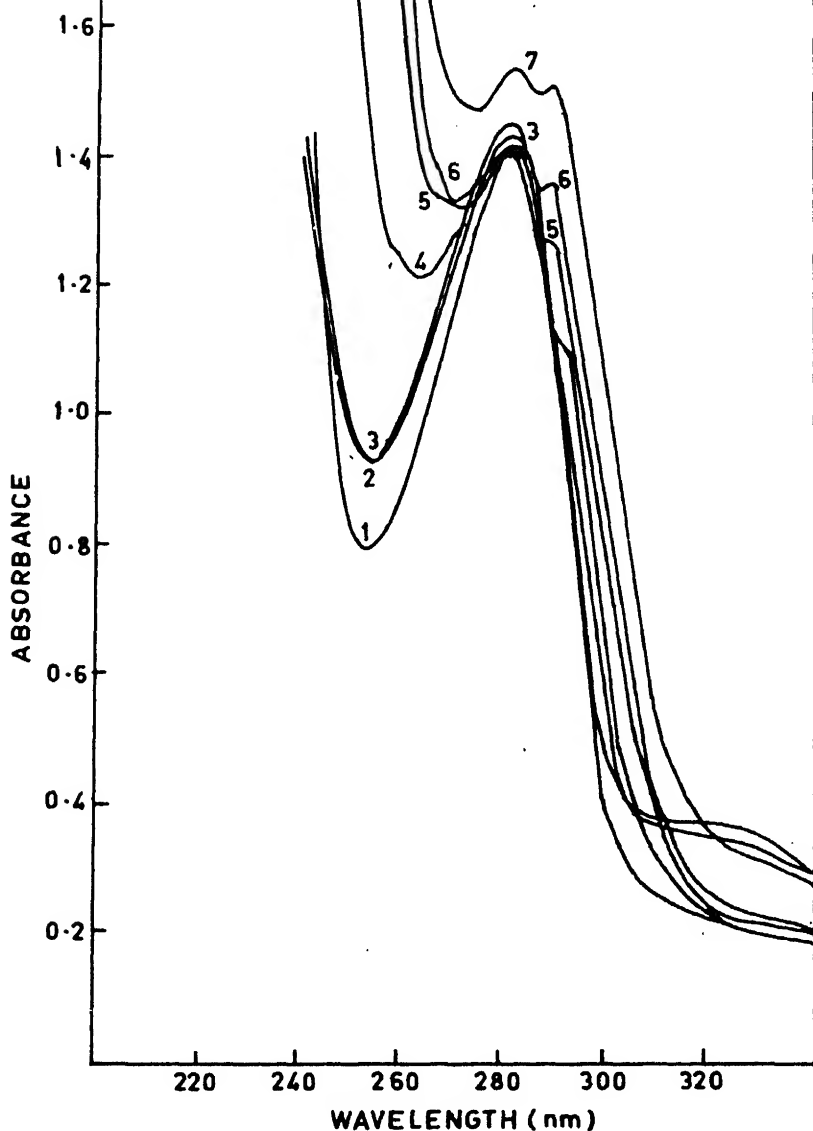


Figure 4. Effect of pH on the turbidity of sunflower 11S protein.

### *Fluorescence spectra*

At pH 8.1, the 11S protein gave a fluorescence emission spectrum with a maximum at 325 nm (figure 6). Quenching of fluorescence intensity and a red shift in the maximum occurred as the pH was increased. The effects were pronounced in the pH range 10.9–11.7. The maximum shifted from 325 nm at pH 8.1 to 340 nm at pH 12.6. The quenching of fluorescence intensity could be due to the exposure of tryptophan residues to the polar environment from the interior hydrophobic environment (Teale, 1960). The quenching could also result from the transfer of resonance energy from indole groups of tryptophan residues to ionized phenol groups of tyrosine residues (Brand and Withold, 1967). The observed red shift in the emission maximum would suggest denaturation of the protein (Teale, 1960).

Turbidity and fluorescence intensity decreased in the pH range 7.0–9.0, even though there was no dissociation of the protein in this pH range. Both ultracentrifugation and PAGE experiments showed the dissociation of the 11S protein at alkaline pH. Decrease in turbidity with increase in pH could also possibly be due to dissociation of the protein. Increase in viscosity (fluorescence quenching and the red shift in emission maximum) could be taken as evidence of denaturation of the protein. Thus at alkaline pH, as in acid pH, sunflower 11S protein dissociated and denatured.



**Figure 5.** Effect of pH on the absorption spectrum of sunflower 11S protein.

(1), pH 7.4; (2), pH 8.0; (3), pH 9.3; (4), pH 10.5; (5), pH 11.3; (6), pH 12.2; (7), pH 12.8.

function of pH, a sigmoidal curve was obtained in each case. Therefore, it was possible to calculate from these data the fraction of the protein dissociated/denatured as a function of pH (Tanford, 1968). The following equation was used:

$$Y = Y_N(1 - \alpha) + \alpha Y_D,$$



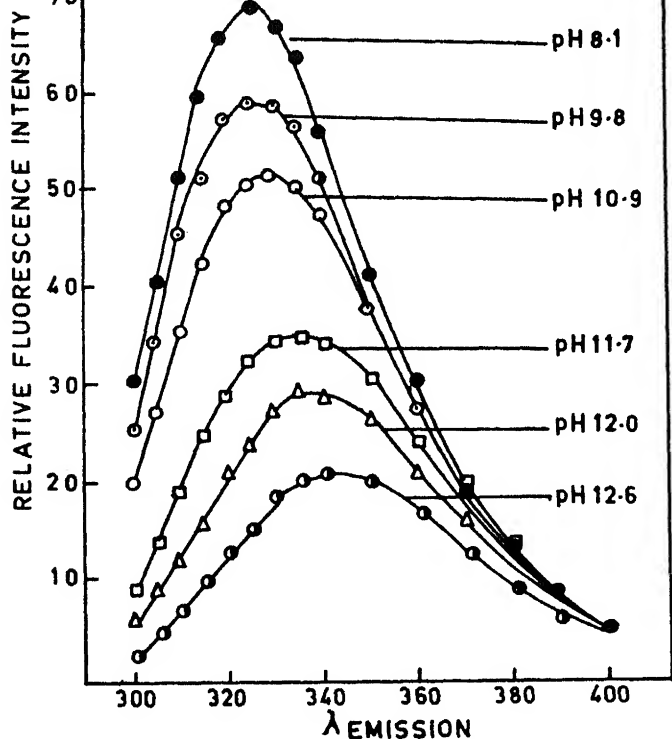


Figure 6. Effect of pH on the fluorescence emission spectrum of sunflower 11S protein. Numbers refer to pH of measurement.

where  $Y$  is the measured value of the parameter (viscosity, turbidity, fluorescence intensity) at a particular pH;  $Y_N$ , the value of the native protein;  $Y_D$ , that of the dissociated/denatured protein and  $\alpha$ , the fraction of the protein dissociated/denatured. The values characteristic of the native protein were obtained from the plateau region values between pH 7.0 and 8.0, and those of the dissociated/denatured protein from the plateau region values between pH 12.0 and 13.0.

The  $\alpha$  values calculated thus are given in figure 7 as a function of pH. The values obtained from the 3 different techniques did not fit the same curve. In protein denaturation studies it is normally assumed that if data from different techniques fit the same curve of  $\alpha$  vs pH, it is indicative of a two-state process (Tanford, 1968). In such a process the native protein is directly converted into the denatured product without any intermediate species.

From figure 7, it may be seen that turbidity decrease (indicative of dissociation of the protein) occurred at a lower pH value than that at which fluorescence intensity and viscosity changes occurred. If viscosity change can be taken as indicative of protein denaturation, it occurred at a much higher pH value. For instance from turbidity data  $\alpha$  had a value of 0.9 at pH 11.5. At the same pH,  $\alpha$  from viscosity data

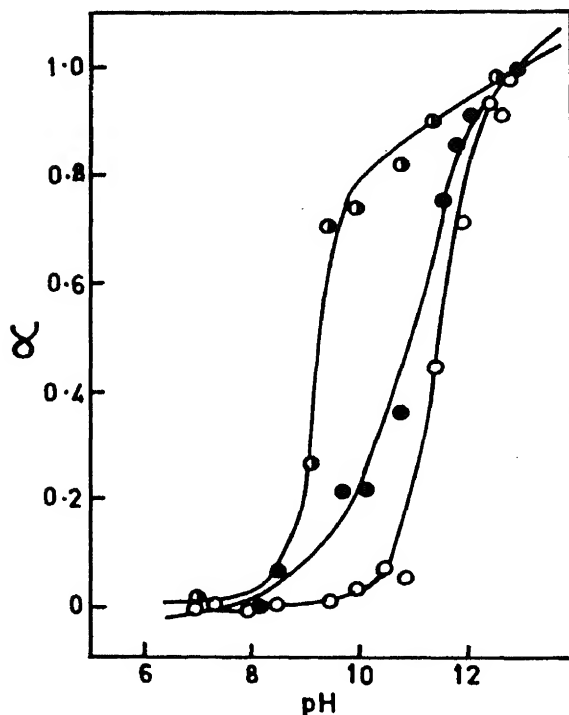


Figure 7. Fraction ( $\alpha$ ) of sunflower 11S protein dissociated/denatured as a function of pH. (○), From  $n_{red}$ ; (●), from turbidity; (●), from fluorescence intensity at 325 nm.

had a value of 0.5 (figure 7). One explanation could be dissociation occurred first and then denaturation later; the two phenomena do not occur simultaneously.

It is not surprising that the data from fluorescence spectra and viscosity do not fit the same curve. Fluorescence quenching is not due to denaturation alone. The (dissociated) phenolic groups also contribute to quenching.

A comparison of the effect of low pH and high pH on the sunflower 11S indicates the following: (i) dissociation and denaturation of the protein occur at both low and high pH; (ii) when the measured property is plotted as a function of pH, a sigmoidal curve is obtained in the alkaline pH range whereas a minimum (or a maximum) is observed in the acid pH range and (iii) in the alkaline pH dissociation/denaturation of the protein goes to completion as the pH is increased and it does not go to completion in acid pH. Below pH 2.5 reaggregation/refolding of the protein occurs.

## References

- Sastry, M. C. S. (1985) *Interactions of Sunflower Proteins*, Ph.D. thesis, University of Mysore, Mysore.
- Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry* (New York: Academic Press).
- Schwenke, K. D., Schultz, M. and Linow, K. J. (1975) *Die Nahrung*, **19**, 817.
- Sripad, G. and Narasinga Rao, M. S. (1987) *J. Agric. Food Chem.*, (in press).
- Tanford, C. (1968) *Adv. Protein Chem.*, **23**, 121.
- Teale, F. W. J. (1960) *Biochem. J.*, **76**, 381.
- Wang, K. L. and Hamlin, L. M. (1974) *Biochemistry*, **13**, 2578.

# Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase $\alpha$ from embryonic chicken brain

IRA SIMET\*, SATYAJIT RAY and SUBHASH BASU†

Department of Chemistry, Biochemistry, Biophysics and Molecular Biology Program, University of Notre Dame, Notre Dame, Indiana 46556, USA

\*Present address: Department of Chemistry, University of Northern Iowa, Cedar Falls, Iowa 50614, USA

**Abstract.** DNA polymerase- $\alpha$  from embryonic chicken brain was resolved on DEAE-cellulose into 3 component activities that remained distinct upon rechromatography. Product formation by each activity required exogenously added template-primer DNA, all 4 deoxynucleoside triphosphates, and a divalent metal cation. Each form incorporated [ $^3\text{H}$ ]-dTTP or [ $^3\text{H}$ ]-dCTP into a high molecular weight product that was identified as DNA by its chromatographic behavior and its sensitivity to DNase. High ionic strength, N-ethylmaleimide, and the polymerase- $\alpha$ -specific inhibitor aphidicolin inhibited each activity; the apparent  $K_i$  value of aphidicolin was 3.0  $\mu\text{M}$  in each case. Based on these results, the 3 activities were identified as multiple forms of DNA polymerase- $\alpha$ . Experiments using embryonic chicken brains of various ages indicated that polymerase- $\alpha_1$  and polymerase- $\alpha_3$  reached maximal activity in 9-day-old embryos, while polymerase- $\alpha_2$  activity was elevated at a slightly later developmental stage. Using poly(dC) as template, high primase activity was detected in polymerase- $\alpha_1$  fractions.

**Keywords.** DNA polymerase- $\alpha$ ; aphidicolin inhibition; primase; N-ethylmaleimide inhibition; DNA chain initiation.

## Introduction

DNA polymerase- $\alpha$  (pol- $\alpha$ ) has been the focus of considerable attention because of its apparent involvement in cellular replicative DNA synthesis. In many studies of pol- $\alpha$  techniques used for partial purification of the enzyme have yielded reproducible multiphasic activity profiles suggestive of enzyme heterogeneity. While such results often were dismissed as artifactual, improved characterizations of the resolved activities revealed distinctions among their properties inconsistent with artificial separations of single enzymatic activities. Consequently, heterogeneity has been accepted as a feature characteristic of pol- $\alpha$ 's (Hubscher, 1983).

Two forms of pol- $\alpha$  were first identified in extracts of calf thymus (Mompalmer *et al.*, 1973). Subsequent investigations using this tissue described 3 (Yoshida *et al.*, 1974; Masaki and Yoshida, 1978) or 4 (Holmes *et al.*, 1974) pol- $\alpha$ 's. Multiple forms of pol- $\alpha$  also have been found in rat liver (Holmes *et al.*, 1974), mouse myeloma (Hachmann and Lezius, 1975; Matsukage *et al.*, 1976; Chen *et al.*, 1979), *Drosophila* embryos (Brakel and Blumenthal, 1977, 1978), yeast (Chang, 1977; Wintersberger, 1978), HeLa cells (Ono *et al.*, 1978, 1979; Lamothe *et al.*, 1981), mouse mastocytoma (Bieri-Bonniot and Schurch, 1978), wheat embryos (Castroviejo *et al.*, 1979), human

†To whom correspondence should be addressed.

Abbreviations used: Pol- $\alpha$ , Polymerase- $\alpha$ ; PEG, polyethylene glycol; 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PMSE, phenylmethyl-

neuroblastoma IMR-32 cells (Bhattacharya *et al.*, 1981), and IMR-32 cells (Takada *et al.*, 1982). The detection of multiple forms in clonal cell lines was particularly significant because it eliminated the possibility that multiple forms of pol- $\alpha$  in tissues were contributed by different cell types within the tissues.

Despite the abundance of information regarding the heterogeneity of pol- $\alpha$ , no relationship between multiple forms and cell or tissue development has been established. Approaches to this question have been hindered by the static natures (complete differentiation or continuous growth) of most of the enzyme sources used. The growth dependent variation in total extractable pol- $\alpha$  activity (Simet, 1983) prompted this investigation of multiple forms of pol- $\alpha$  in developing embryonic chicken brain.

The multiple forms of DNA polymerases from various eukaryotic sources appear to contain a primase activity in addition to other peptides with unknown functions. The primase subunit closely associated with DNA polymerase- $\alpha$  initiates RNA primers before any DNA chain initiation in mouse tumor cells (Yagura *et al.*, 1982; Kozu *et al.*, 1986), *Drosophila* (Kaguni *et al.*, 1984), *Xenopus laevis* (Shioda *et al.*, 1982), human KB cells (Wang *et al.*, 1984), Yeast (Plevani *et al.*, 1985), calf thymus (Grosse and Kraus, 1985; Holmes *et al.*, 1985), and human IMR-32 neuroblastoma cells (Takada *et al.*, 1986a, b).

## Materials and methods

The following materials were acquired from the indicated commercial source: poly(rA)·(dT)<sub>12-18</sub> (20:1), P-L Biochemicals, Milwaukee, Wisconsin, USA; DE-23 diethylaminoethylcellulose, Whatman Ltd., Springfield, England; polyethylene glycol (PEG; average molecular weight 20,000), and *Streptomyces griseus* Pronase (type VI), Sigma Chemical Company, St. Louis, Missouri, USA; Sephadex G-100 and blue dextran 2000, Pharmacia Fine Chemicals, Piscataway, New Jersey, USA; bovine pancreatic ribonuclease (RNase), Worthington Biochemicals, Freehold, New Jersey, USA; [methyl-<sup>3</sup>H]-thymidine 5'-monophosphate (dTMP; 18 Ci/mmol), Amersham Corporation Arlington Heights, Illinois, USA; [<sup>3</sup>H]-DNA (IMR-32), gift from Dr. Prabir Bhattacharya, Notre Dame, Indiana, USA; diphenylamine, J. T. Baker Chemical Company, Phillipsburg, New Jersey, USA; and aphidicolin, gift from the Drug Synthesis and Chemistry Branch, Department of Therapeutic Pharmaceuticals, Division of Cancer Treatment of the National Cancer Institute, Bethesda, Maryland, USA. All other reagents were of the highest purity available.

### Assays for DNA polymerases

The complete assay incubation mixture for pol- $\alpha$  contained the following components in a total volume of 0.1 ml; 10  $\mu$ g of activated calf thymus DNA; 50 mM Tris-HCl, pH 8.5; 5 mM MgCl<sub>2</sub>; 20 mM KCl; 5 mM 2-mercaptoethanol (2-ME); 50  $\mu$ g of bovine serum albumin (BSA); 50  $\mu$ M [<sup>3</sup>H]-dTTP [specific activity, 120-200 cpm/pmol], dATP, dCTP, and dGTP; and enzyme (10-25  $\mu$ g of protein as estimated by the method of Lowry *et al.* (1951)). The mixtures were incubated at 37°C for 30 min, after which the reaction was stopped by adding 50  $\mu$ g of herring

Whatman GF/C borosilicate glass discs in a Millipore apparatus. The discs were dried at 80°C, and [<sup>3</sup>H]-dTTP incorporation was quantitated using a toluene-based mixture in a Beckman LS-3300-T liquid scintillation counter.

For pol- $\beta$ , the above reaction mixture was used except that the 2-ME was omitted and 10 mM N-ethylmaleimide was added. For pol- $\gamma$ , the activated calf thymus DNA was replaced with 2.5  $\mu$ g of poly(rA)·(dT)<sub>12-18</sub>, the KCl concentration was increased to 50 mM, the nonradioactive dNTPs were omitted, and 50 mM K-PO<sub>4</sub>, pH 8.5, was added. In assays for terminal deoxynucleotidyltransferase, 2.5  $\mu$ g of poly(dA) replaced the activated DNA; 50 mM Tris-HCl, pH 7.0, replaced 50 mM Tris-HCl, pH 8.5; 0.5 mM MnCl<sub>2</sub> replaced the MgCl<sub>2</sub>; KCl and the unlabelled dNTPs were omitted; and [<sup>3</sup>H]-dGTP (specific activity, 200 cpm/pmol) was used as substrate. The complete assay mixture for pol- $\gamma$  contained the following components in a total volume of 0.1 ml: 2.5  $\mu$ g of poly(rA)·(dT)<sub>12-18</sub>; 50 mM Tris-HCl, pH 8.5; 5 mM MgCl<sub>2</sub>; 50 mM KCl; 5 mM 2-ME; 50  $\mu$ g BSA; 50  $\mu$ M [<sup>3</sup>H]-dTTP (specific activity, 120 to 250 cpm/pmol); 50 mM potassium phosphate (K-PO<sub>4</sub>), pH 8.5 (to suppress pol- $\beta$  activity); and 10–25  $\mu$ g enzyme protein [estimated according to Lowry *et al.* (1951)]. The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 50  $\mu$ g herring sperm DNA and ice-cold 10% (w:v) trichloroacetic acid containing 0.1 M sodium pyrophosphate. After chilling in ice for 20 min, the reaction mixtures were filtered through DE-81 diethylaminoethyl paper filter disks (Whatman Ltd.) in a Millipore apparatus. The disks were dried and counted as described for pol- $\alpha$  and pol- $\beta$ . Counting efficiency was approximately 10%.

Reaction rates observed with each resolved activity were proportional to all protein concentrations used and remained constant with incubation times up to 45 min.

### *Separation of multiple forms of DNA polymerase- $\alpha$*

A portion of sonicated (100,000 g) supernatant [50 mM Tris-HCl buffer, pH 8.0; 6 mM KCl; 2 mM MgCl<sub>2</sub>; 5 mM dithiothreitol (DTT); 1 mM ethylenediaminetetraacetate (EDTA); 0.5 mM phenyl methyl sulphonylfluoride (PMSF)] prepared from 9-day-old embryonic chicken brains, containing 10 to 14 mg protein, was applied to a DEAE-cellulose column (2×11 cm) that had been equilibrated with 50 mM K-PO<sub>4</sub>, pH 7.6, containing 1 mM DTT. After nonadsorbed protein had been washed from the column with 100 ml of the equilibration buffer, the multiple forms of pol- $\alpha$  were eluted with a 40-ml linear buffer gradient of 50–350 mM K-PO<sub>4</sub>, pH 7.6, containing 1 mM DTT. A flow rate of 1 ml per min was maintained while fractions of approximately 1 ml were collected. Each fraction was assayed for DNA-polymerizing activities as described above. To fractions containing pol- $\alpha$  activity were added 15  $\mu$ g herring sperm DNA. These fractions then were made 50% (v:v) in glycerol or 40% (v:v) in ethylene glycol before storage at -18°C.

### *Rechromatography of multiple forms of DNA polymerase- $\alpha$*

Pol- $\alpha$  containing fractions to be rechromatographed were used immediately after

resolution and therefore contained no added DNA, glycerol, or ethylene glycol. After the fractions had been diluted with  $\text{H}_2\text{O}$  to adjust their  $\text{K-PO}_4$  concentrations to 50 mM, each was applied separately to a DEAE-cellulose column ( $1 \times 8$  cm) prepared as described above. The column was washed and developed as described above except that buffer volumes were halved. Fractions were assayed for pol- $\alpha$  activity.

### *Isolation of reaction products*

Incubations using [ $^3\text{H}$ ]-dTTP-containing pol- $\alpha$  reaction mixtures were stopped by addition of EDTA, pH 7.6, to a final concentration of 10 mM. The mixtures were pooled and applied to a Sephadex G-100 column ( $1 \times 52$  cm; void volume, 17 ml, determined from an elution profile of blue dextran 2000) that had been equilibrated with 15 mM NaCl. The column was developed with the same eluent at a flow rate of 0.2 ml per min. Fractions (1 ml each) were assayed for radioactivity by spotting aliquots on GF/B borosilicate glass fiber disks (Whatman Ltd.) and quantitating [ $^3\text{H}$ ]-label as described for DNA polymerase assays. Void volume fractions were pooled and concentrated by dialysis against solid PEG.

### *Paper chromatography of reaction products*

Samples containing concentrated reaction products were spotted on SG-81 silica gel-impregnated paper (Whatman Ltd.) and dried. [ $^3\text{H}$ ]-DNA from IMR-32 (10  $\mu\text{g}$ ), [ $^3\text{H}$ ]-dTTP (approximately 1 nmol), and [ $^3\text{H}$ ]-dTTP (approximately 1 nmol) were run concurrently as standards. Chromatograms were developed in an ascending manner with 95% ethanol/1 M ammonium acetate, pH 7.3 (7:3, v:v). The chromatogram was dried and each lane was cut into 1" squares. Radioactivity was quantitated using the toluene based mixture described for DNA polymerase assays.

### *Enzymatic hydrolysis of reaction products*

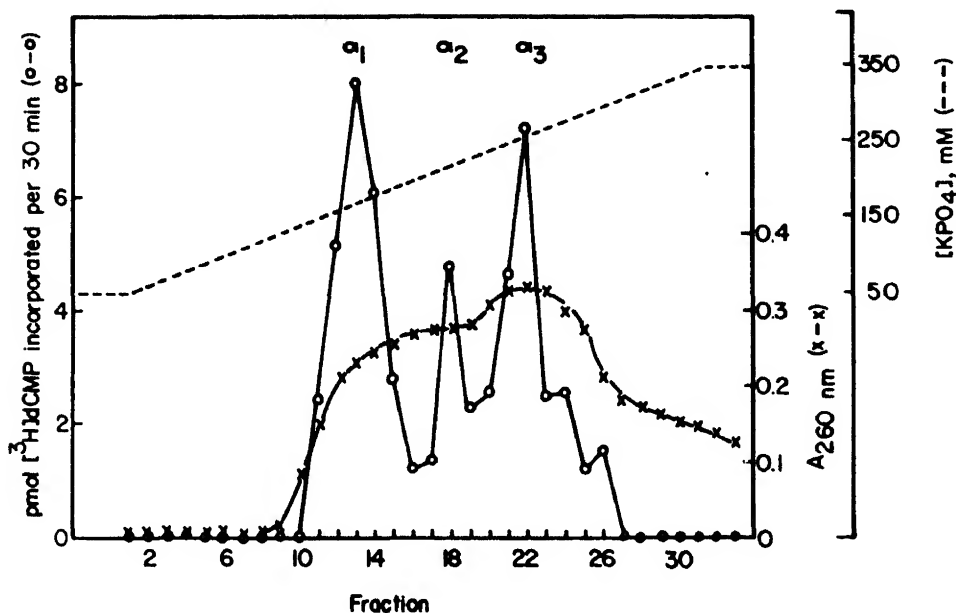
The complete reaction mixtures contained the following components in a final volume of 0.1 ml: reaction product, 400–900 cpm; Tris-HCl, pH 7.5, 50 mM;  $\text{MgCl}_2$ , 5 mM;  $\text{CaCl}_2$ , 5 mM (used only with Pronase); and enzyme, 1–10  $\mu\text{g}$  per ml. The mixtures were incubated at 37°C for 5–30 min, and reactions were stopped by the addition of 50  $\mu\text{g}$  herring sperm DNA and ice-cold 10% (w:v) trichloroacetic acid containing 0.1 M sodium pyrophosphate. The remaining acid-precipitable product was quantitated on GF/C disks as described for DNA polymerase assays.

### *Analytical Methods*

Protein was quantitated according to Lowry *et al.* (1951) using BSA as a standard. The DNA contents of protein-free mixtures were estimated from absorbance at 260 nm after the mixtures had been heated at 90°C for 10 min to denature the DNA; an absorbance of 20.0 was assumed to represent 1 mg DNA per ml. The DNA

### Separation of multiple forms of DNA polymerase- $\alpha$

Chromatography on DEAE-cellulose resolved several DNA-polymerizing activities in embryonic chicken brain extracts. The pol- $\beta$  resistant to N-ethylmaleimide (NEM) was not adsorbed to the resin and was recovered in the wash fraction. Pol- $\gamma$ , identified by its efficient copying of poly (rA)·(dT)<sub>12-18</sub>, was eluted at approximately 140 mM K-PO<sub>4</sub>. Three other forms of DNA-polymerizing activity also were identified. Pol- $\alpha_1$  was eluted at 160–165 mM K-PO<sub>4</sub>, pol- $\alpha_2$  at 220 mM K-PO<sub>4</sub>, and pol- $\alpha_3$  at 260–270 mM K-PO<sub>4</sub> (figure 1). Concentrations of K-PO<sub>4</sub> up to 500 mM failed to elute any additional enzymatic activities. Extracts from both 9- and 13-day-old embryonic chicken brains yielded this chromatographic profile. Separation of the 3 forms resulted in a 30- to 40-fold purification of the DNA polymerase activities, with a total yield of 60–70% (table 1).



**Figure 1.** Resolution of multiple forms of DNA pol- $\alpha$  from 9-day-old embryonic chicken brain. An aliquot of 9-day-old embryonic chicken brain sonicated supernatant containing 14 mg protein was applied to a DEAE-cellulose column (2×11 cm) that had been equilibrated with 50 mM K-PO<sub>4</sub>, pH 7.6, containing 1 mM DTT. After nonadsorbed protein had been washed from the column with the same buffer, the 3 pol- $\alpha$ s were eluted with a 40-ml linear buffer gradient of 50–350 mM K-PO<sub>4</sub>, pH 7.6, containing 1 mM DTT. Fractions of 1.1 ml were collected and assayed for pol- $\alpha$  activity. (o), Pol- $\alpha$  activity (in picomoles of [<sup>3</sup>H]-dCMP incorporated into activated DNA per 30 min); (x), absorbance at 260 nm; (---), K-PO<sub>4</sub> concentration.

A fourth activity, infrequently observed, was eluted at 195 mM K-PO<sub>4</sub>. In each instance, the presence of this activity was accompanied by a decline in at least one of



Table 1. Separation of DNA polymerases from 9-day-old embryonic chicken brain.

Fraction	Volume (ml)	Total units	Units per mg protein	Yield (%)
Homogenate	6.8	6195	71.6	100
Sonicate	6.8	8969	108.0	145
Sonicated supernatant	5.1	5600	195.8	90
Pellet	1.0	774	35.6	12
Pol- $\alpha_1$	3.3	3058	2315.0	49
Pol- $\alpha_2$	1.1	757	1146.3	12
Pol- $\alpha_3$	2.2	1888	2861.0	30
Total DEAE fractions		5703		91

Activities were measured using the activated calf thymus DNA-based assay for pol- $\alpha$  described in 'materials and methods'. Protein was quantitated according to Lowry *et al.* (1951).

Enhancement of purification by selective enrichment of pol- $\alpha$  activity was attempted using a PEG precipitation procedure (Duguet *et al.*, 1978). The sonicated supernatant was made 2 M in NaCl and a high molecular weight protein fraction was precipitated by addition of PEG to 10% (w/v). The precipitate was collected, redissolved, and dialyzed to remove NaCl and PEG. This procedure reduced the yields of pol- $\alpha$  by 70–90% and therefore was not included in subsequent preparations.

Extract preparation in buffers of pH 8.5 or in the presence of EDTA or sucrose had no effect on yields or elution profiles. Also, no differences in elutive strengths or relative peak areas accompanied omission of the serine protease inhibitor PMSF from the homogenizing buffers. All 3 forms of enzymatic activity were present in both sonicated and unsonicated homogenates.

### *Rechromatography of multiple forms of DNA polymerase- $\alpha$*

Each activity yielded a single major peak of activity upon rechromatography on DEAE-cellulose (figure 2). No evidence of any significant secondary activities was apparent. The K- $\text{PO}_4$  concentrations required for elution of each of the forms were unchanged upon rechromatography. The recoveries of enzyme units applied were 78% for pol- $\alpha_1$ , 59% for pol- $\alpha_2$ , and 52% for pol- $\alpha_3$ .

### *Stability of multiple forms of DNA polymerase- $\alpha$*

DNA-polymerizing activity in the supernatant was fairly stable at 4°C. At -70°C, the resolved activities were completely stable for 2–3 weeks in the presence of 0.1% 8K PEG and 20% glycerol. Addition of 2 mM MgCl<sub>2</sub> or 0.5 mM PMSF contributed little additional stabilization. However, the 3 forms individually were markedly unstable at 4°C with nearly total loss of activity within 20 h in the absence of 0.1% PEG. Addition of BSA to the fractions had no effect on the life-times of the activities. Virtually all of the initial enzymatic activities in the supernatant could be recovered

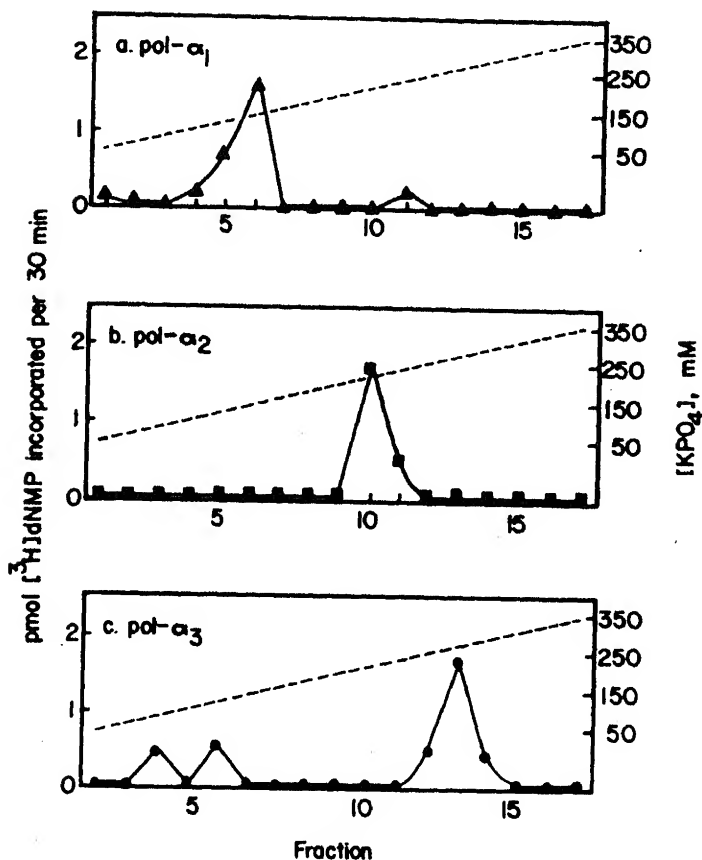


Figure 2. Rechromatography on DEAE-cellulose of multiple forms of DNA pol- $\alpha$  from embryonic chicken brain. Fractions containing multiple forms of embryonic chicken brain pol- $\alpha$  were diluted with  $H_2O$  to adjust their  $K-PO_4$  concentrations to 50 mM and were applied separately to a DEAE-cellulose column ( $1 \times 8$  cm) that had been equilibrated with 50 mM  $K-PO_4$ , pH 7.6, containing 1 mM DTT. The column was washed and developed as described in figure 1 except that a 20-ml gradient volume was used. (a), Pol- $\alpha_1$   $\Delta$ ; (b), pol- $\alpha_2$   $\blacksquare$ ; (c), pol- $\alpha_3$   $\bullet$ ; (---),  $K-PO_4$  concentration.

or 40% (v:v) in ethylene glycol prior to storage. Moreover, addition of herring sperm DNA to a concentration of 15  $\mu g$  per ml helped maintain initial activity levels in the glycerolic fractions. The additional stabilization was not due to the increased concentration of potential substrate DNA in the assay mixtures, as the endogenous activities of the fractions remained negligible.

#### Requirements for enzymatic activity

The requirements for product formation were found to differ little among the 2.6

Table 2. Requirements for enzymatic activity.

Reaction mixture	[ <sup>3</sup> H]-dNMP incorporated		
	pol- $\alpha_1$	pol- $\alpha_2$	pol- $\alpha_3$
	(pmol/mg protein/h)		
Complete	480.8	465.6	1258.0
— Activated DNA	0.0	0.0	0.0
— KCl	0.0	283.8	402.8
— MgCl <sub>2</sub>	446.8	454.8	359.4
— MgCl <sub>2</sub> , + MnCl <sub>2</sub> (1 mM)	257.4	113.0	375.4
— MgCl <sub>2</sub> , + EDTA (10 mM)	38.6	0.0	0.0
— 2-ME	271.4	204.8	568.0
— 2-ME, + NEM (10 mM)	29.2	139.6	0.0
— BSA	43.2	0.0	127.6

The complete incubation mixtures contained the following components in a total volume of 0.1 ml: 20  $\mu$ g activated calf thymus DNA; 20 mM KCl; 5 mM MgCl<sub>2</sub>; 50 mM Tris-HCl, pH 8.5; 5 mM 2-ME; 50  $\mu$ g BSA; and 50  $\mu$ M [<sup>3</sup>H]-dTTP<sup>2</sup> and each unlabelled dNTP.

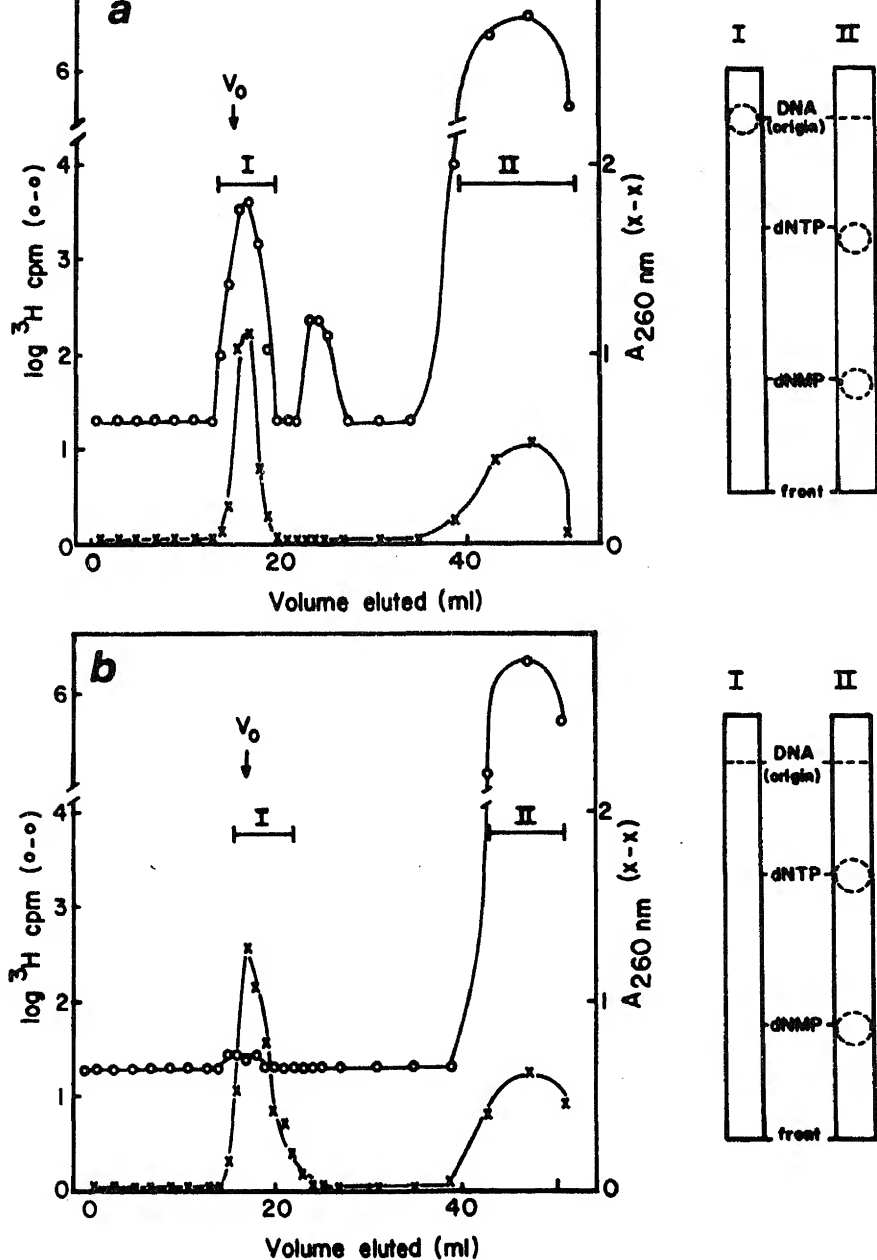
enzymatic activities on a divalent metal cation was suggested by their complete sensitivities to EDTA, although similar decreases in DNA synthesis could not be obtained by omission of exogenously added MgCl<sub>2</sub>. Each enzymatic form was most active with MgCl<sub>2</sub>, and addition of MnCl<sub>2</sub> to reaction mixtures prepared without MgCl<sub>2</sub> failed to restore optimal activity.

#### *Characterization of [<sup>3</sup>H]-labelled products synthesized by the multiple forms of DNA polymerase- $\alpha$*

Two components with strong absorbances at 260 nm were resolved by Sephadex G-100 chromatography of the reaction mixtures (figure 3a). Over 95% of the initial radioactivity of the reaction mixtures was recovered in the included component, which was identified as a mixture of deoxynucleoside mono- and tri-phosphates by paper chromatography. The excluded material contained radioactivity only if enzyme had been added to the reaction mixture; enzyme-free control reaction mixtures yielded a non-radioactive excluded fraction of material absorbing at 260 nm (figure 3b). Like [<sup>3</sup>H]-DNA from IMR-32 cells, the labelled products failed to move from the origin upon paper chromatography on SG-81 paper in ethanol/ammonium acetate. The excluded fractions therefore were identified as DNA into which [<sup>3</sup>H]-labelled dNMP residues had been incorporated by the enzymatic activities.

Following concentration, each of the excluded components was treated with hydrolytic enzymes. DNase rendered 70–80% of the [<sup>3</sup>H]-labelled materials acid-soluble after 5 min at 37°C; only slightly more solubilization was effected by longer incubation (table 3). Neither RNase nor Pronase solubilized more than 5–7% of the [<sup>3</sup>H]-labelled materials, even at 10-fold greater concentrations and with longer incubations.

#### *Inhibition of multiple forms of DNA pol- $\alpha$ by high ionic strength*



**Figure 3.** Isolation and partial characterization of  ${}^3\text{H}$ -labelled products synthesized by DNA pol- $\alpha$ , from embryonic chicken brain. Pol- $\alpha$  assay mixtures stopped by the addition of EDTA were chromatographed on Sephadex G-100, using 15 mM NaCl as the eluent. Excluded peaks (I) and included peaks (II) absorbing at 260 nm were pooled as indicated by the bars. After concentration of the excluded material, samples of peaks I and II were chromatographed on SG-81 paper in an ascending fashion using 95% ethanol/1 M ammonium acetate, pH 7.3 (7:3, v: v), (a), Pol- $\alpha$  product; (b), enzyme-free reaction mixture.  $(\text{O})$ , acid-insoluble  ${}^3\text{H}$  counts per 0.1 ml;  $(x)$ , absorbance at 260 nm. The resulting profiles

Enzyme added	Concentration	Rendered acid-soluble		
		Pol- $\alpha_1$	Pol- $\alpha_2$	Pol- $\alpha_3$
			%	
None		0	0	0
DNase I	1 $\mu\text{g/ml}$	72	82	77
Pancreatic RNase	10 $\mu\text{g/ml}$	7	6	2
Pronase	10 $\mu\text{g/ml}$	4	3	5

Partially purified [ $^3\text{H}$ ]-labelled reaction products were concentrated by dialysis against solid PEG and then were treated with hydrolytic enzymes for 15 min at 37°. Total acid-precipitable materials in the untreated samples were: pol- $\alpha_1$  product, 442 cpm; pol- $\alpha_2$  product, 908 cpm; pol- $\alpha_3$  product, 817 cpm.

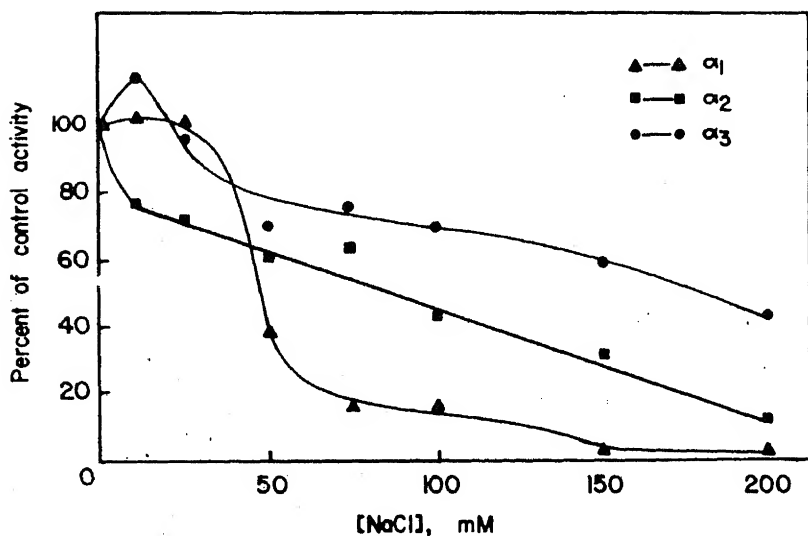
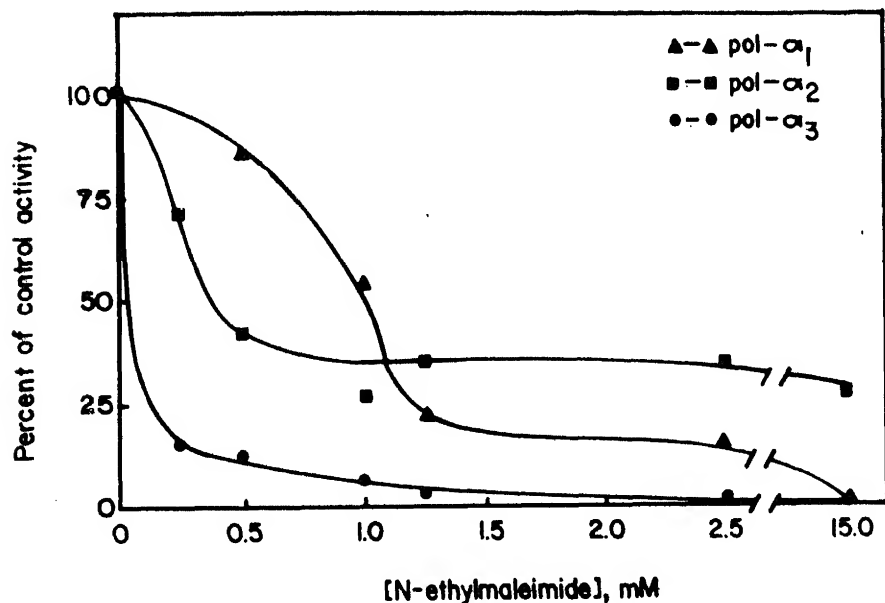


Figure 4. Inhibition by NaCl of multiple forms of DNA pol- $\alpha$  from embryonic chicken brain. One hundred percent activity represents the following values, in picomoles of [ $^3\text{H}$ ]-dTMP incorporated into activated DNA per mg protein per h. ( $\blacktriangle$ ), 650 cpm for pol- $\alpha_1$ ; ( $\blacksquare$ ), 500 cpm for pol- $\alpha_2$ ; ( $\bullet$ ), 1200 cpm for pol- $\alpha_3$ .

Pol- $\alpha_1$  activity was abolished completely by 150 mM NaCl. At 200 mM NaCl, pol- $\alpha_2$  and pol- $\alpha_3$  were inhibited 85 and 50%, respectively. For each form, 50% of maximal inhibition was observed at 50 mM NaCl.

A mixture of the 3 forms was inhibited 50% by 25 mM K- $\text{PO}_4$  and 100% by 100 mM K- $\text{PO}_4$ ; in contrast, pol- $\beta$ , obtained from the wash fraction during DE-23 chromatography, was completely inhibited by 25 mM K- $\text{PO}_4$ .

The effects of NEM on the enzymatic activities were assessed using assay mixtures lacking 2-ME to avoid neutralization of the NEM. All 3 forms were inhibited by NEM (figure 5). Pol- $\alpha_3$  activity was the most sensitive, exhibiting 50% inhibition at 0.25 mM NEM and inhibition at 2.5 mM NEM. Pol- $\alpha_2$  and pol- $\alpha_3$  activities were inhibited 100% by 15 mM NEM, while pol- $\alpha_2$  could not be totally inhibited by any NEM concentration up to 15 mM. All 3 enzymatic activities were completely abolished by pre-incubation with 2.5 mM NEM at 4°C for 10 min (data not shown).



**Figure 5.** Inhibition by NEM of multiple forms of DNA pol- $\alpha$  from embryonic chicken brain. Assay mixtures were prepared without 2-ME to avoid neutralization of the NEM. One hundred percent activity presents the following values picomoles of [ $^3$ H]-dTMP incorporated into activated DNA per mg protein per h. ( $\Delta$ ), 660 cpm for pol- $\alpha_1$ ; ( $\blacksquare$ ), 500 cpm for pol- $\alpha_2$ ; ( $\bullet$ ), 2240 cpm for pol- $\alpha_3$ .

#### *Inhibition of multiple forms of DNA polymerase $\alpha$ by aphidicolin*

In initial experiments with aphidicolin, aliquots from a stock solution of 400  $\mu$ g aphidicolin per ml of 25% (v:v) aqueous dimethyl sulphoxide were added to assay mixtures to yield the desired final concentrations. Under these conditions, dimethyl sulphoxide had little effect on low levels of DNA syntheses. However, 2.5% DMSO inhibited DNA-polymerizing activities by 20–40% at saturating dCTP concentrations. Similar but less pronounced inhibition of *Xenopus* ovarian pol- $\alpha$  has been reported (Zimmerman *et al.*, 1980). To circumvent this problem, a stock solution of

averaged 7% and did not exceed 13%, and neither  $\text{pol-}\alpha_2$  nor  $\text{pol-}\alpha_3$  was affected.

Under the latter conditions, aphidicolin inhibited each of the 3 activities to similar extents (figure 6a). For each form of  $\text{pol-}\alpha$ , enzymatic activity was measured in the presence of 6 concentrations of aphidicolin and 5 dCTP concentrations; representative data are illustrated. Each activity was reduced 50% by 6–10  $\mu\text{M}$  aphidicolin at 1  $\mu\text{M}$  dCTP and by 20–30  $\mu\text{M}$  aphidicolin at 5  $\mu\text{M}$  dCTP. At 50  $\mu\text{M}$  dCTP, none of the activities was inhibited more than 30% by aphidicolin concentrations up to 30  $\mu\text{M}$ .

In double-reciprocal plots of data from the aphidicolin inhibition experiments, aphidicolin appeared to be a competitive inhibitor of each of the enzymatic activities (Figure 6b). The slopes of the double reciprocal lines, obtained from a line-fitting program based on least squares analysis run on an Apple II computer, were directly proportional to inhibitor concentrations, indicating that the mode of inhibition in each instance was linearly competitive. The  $K_i$  values of aphidicolin, calculated from the double-reciprocal plots, were 2.5  $\mu\text{M}$  for  $\text{pol-}\alpha_1$ , 3.1  $\mu\text{M}$  for  $\text{pol-}\alpha_2$ , and 3.0  $\mu\text{M}$  for  $\text{pol-}\alpha_3$ .

### *Characterization of DNA $\text{pol-}\alpha_1$ -primase complex*

The presence of primase activity in the 3 active DNA  $\text{pol-}\alpha$  forms were tested using poly (dC) template and rGTP as a donor for the primer synthesis followed by DNA chain extension with [ $^3\text{H}$ ]-dGTP (Takada *et al.*, 1986b). It appeared that the column fraction that contained peak activity of DNA  $\text{pol-}\alpha_1$  (tested with ACT-DNA) also had the highest primase activity (figure 7).

## **Discussion**

The association of elevated  $\text{pol-}\alpha$  activity with rapid growth has stimulated the study of this enzyme in developing and differentiating systems. In only one of these systems, *Drosophila* embryos with multiple forms have been described (Brakel and Blumenthal, 1977, 1978). *Drosophila* enzyme III, which sedimented at 9.0.S, was enriched in early embryonic development (Brakel and Blumenthal, 1977), when total extractable  $\text{pol-}\alpha$  activity was at a maximum (Margulies and Chargaff, 1973; Karkas *et al.*, 1975). Conversion of the 9.0.S enzyme to smaller and possibly less active species therefore appeared to accompany a decline in  $\text{pol-}\alpha$  activity throughout development.

To address further the involvement of multiple forms of  $\text{pol-}\alpha$  in development, investigations of embryonic chicken brain  $\text{pol-}\alpha$  were undertaken. Only a microsomal DNA-polymerizing activity that could not be identified unambiguously as  $\text{pol-}\alpha$  had been isolated from this tissue (Smith and Soriana, 1977). The elevation and subsequent decline in  $\text{pol-}\alpha$  levels during embryonic chicken brain development (Simet, 1983) suggested that this source would be well-suited for developmental studies. Previous reports of  $\text{pol-}\alpha$  in whole chicken embryos (Brun *et al.*, 1974; Yamaguchi *et al.*, 1982) had contained some indications, not pursued in detail, that the enzyme might be heterogeneous. In addition, both  $\text{pol-}\beta$  (Stavrianopoulos *et al.*, 1971, 1972a,b) and  $\text{pol-}\gamma$  (Bertozzi *et al.*, 1977; Yamaguchi *et al.*, 1982) from whole

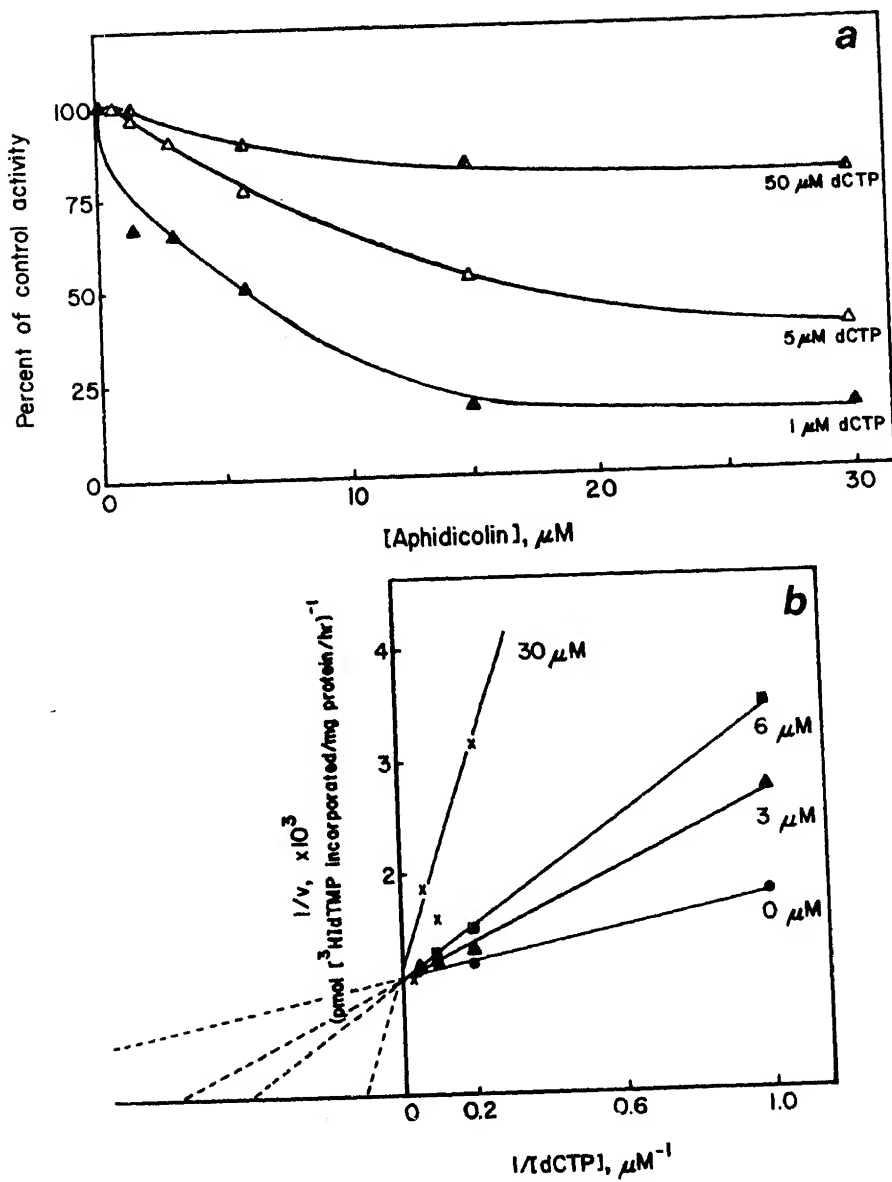
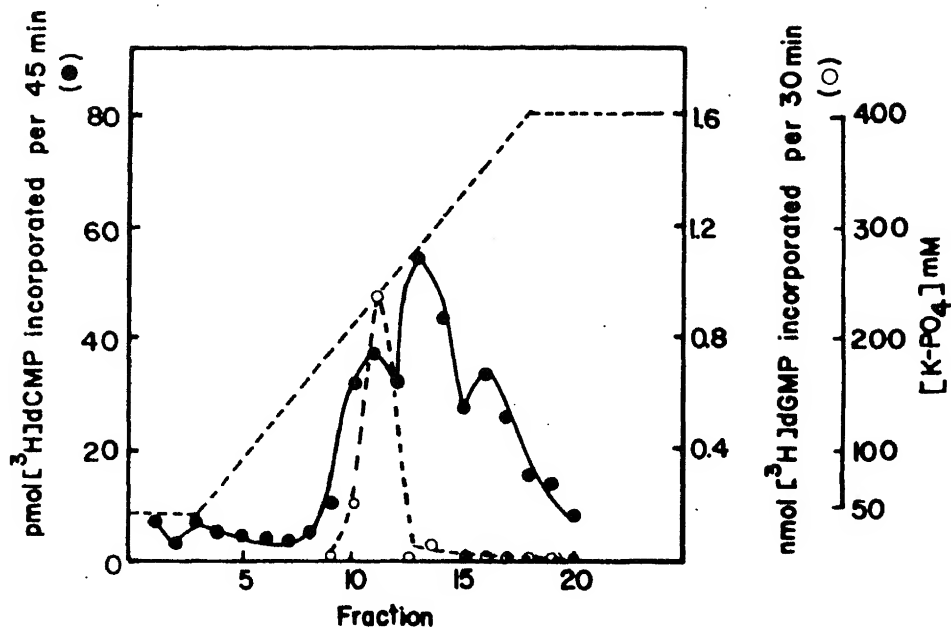


Figure 6. Inhibition by aphidicolin of multiple forms of DNA pol- $\alpha$  from embryonic chicken brain. All assay mixtures were adjusted to 0.25% (v:v) DMSO. Enzymatic activity was measured in the presence of 6 concentrations of aphidicolin and 5 concentrations of dCTP with representative data illustrated.

(a). Pol- $\alpha_1$ . One hundred percent activity represents the following values, in picomoles of [ $^3\text{H}$ ]dCTP incorporated into activated DNA per mg protein per h. ( $\Delta$ ), 550 for 1  $\mu\text{M}$  dCTP; ( $\bullet$ ), 1100 for 5  $\mu\text{M}$  dCTP.





**Figure 7.** Resolution of DNA polymerase-primase complex on DEAE-23. Frozen embryonic brains (9-day-old) were homogenized in 3 volumes of 50 mM Tris-HCl (pH 8.0) buffer containing 6 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 2.5 mM benzamidine, 50 µg/ml soybean trypsin inhibitor, 5 µg/ml each of leupeptin and pepstatin, 1 mM aminoacetoneitrile bisulphate, and 0.1% PEG (M = 8,000). The homogenate was centrifuged at 105,000 g for 2 h. The supernatant containing 40 mg protein was applied to a DEAE-23 a cellulose column (1.5 × 9 cm) preequilibrated with 50 mM potassium phosphate-buffer (pH 7.6) containing 5 mM DTT and 0.1% PEG (8K). After washing of non-adsorbed proteins with the column buffer, primase (○) and DNA polymerase α (●) activities were eluted with a 40 ml linear buffer gradient of 50–400 mM K-PO<sub>4</sub>, pH 7.6, containing 5 mM DTT and 0.1% PEG (8K) at a rate of 1 ml/min. Fractions were collected in 20% glycerol (final concentrations) and were stored at -70° before assay. Primase activity was assayed using poly (dC) template and [<sup>3</sup>H]-dGTP; pol-α was assayed using activated calf thymus and [<sup>3</sup>H]-dCTP. Reaction conditions were the same as described in the text.

embryos had been characterized, facilitating comparisons of any resolved enzymatic species with known activities.

Because pol-α was the predominant DNA-polymerizing activity in 9 to 13-day-old embryonic chicken brain, initial studies were conducted using the tissue at this developmental stage. Attempts to enrich selectively the pol-α contents of embryonic chicken brain extracts by PEG precipitation were unsuccessful, probably because of the extensive loss of the unstable activity during the lengthy dialysis step required for the removal of NaCl and PEG. However, ion-exchange chromatography on DEAE-cellulose successfully resolved pol-β, pol-γ, and 3 additional forms of DNA-polymerizing activity (figure 1). Nonadsorption of pol-β was expected because of its known basicity (Stavrianopoulos et al., 1972a; Simet, 1982) and the elution of pol-

The 3 other activities were eluted at 165, 220, and 265 mM K-PO<sub>4</sub>. To establish that each of these activities was a DNA polymerase, the [<sup>3</sup>H]-labelled products of the enzymatic reactions were isolated by chromatography on Sephadex G-100. The exclusion of the products by the resin (figure 3a) indicated that they were of high molecular weight. Paper chromatography demonstrated that the [<sup>3</sup>H]-label in the excluded fractions did not reside on single deoxynucleotides. The sensitivities of the products to DNase and their resistances to RNase and Pronase (table 3) confirmed that the multiple forms were incorporating [<sup>3</sup>H]-dNMP residues into DNA.

The requirements for enzymatic activity provided additional evidence that the multiple forms were DNA polymerases. All 3 species required a DNA template-primer, all 4 dNTPs, and a divalent metal cation for optimal product synthesis (table 2).

Because heterogeneity of chick embryo pol- $\alpha$  had not been emphasized in previous studies, the possibility that the 3 activities were merely artifactual species was raised. Artificial fractionation of a single enzymatic species by ion-exchange chromatography appeared unlikely because each of the forms was eluted at its initial elutive ionic strength upon rechromatography, and no secondary activities were generated by the second passage over DEAE-cellulose (figure 2). Alternatively, the 3 forms might have represented complexes of a single enzymatic activity with varying amounts of DNA, with complexes of higher DNA content adsorbing more tightly to the resin. DNA had been detected in a DEAE-cellulose-purified *Drosophila* pol- $\alpha$  fraction (Karkas *et al.*, 1975), but retention of the DNA of DNA-protein complexes by DEAE-cellulose at concentrations in excess of 300 mM K-PO<sub>4</sub> had also been reported (Brakel and Blumenthal, 1977). The unchanged elution patterns of the 3 forms upon rechromatography suggested that the initial resolution had not been based on differing DNA contents.

Extensive evidence of sensitivity of pol- $\alpha$ s to proteolysis (Hubscher, 1983) suggested that proteolytic degradation during extraction of a single pol- $\alpha$  might have been responsible for the observed enzymatic heterogeneity. However, omission of PMSF from extraction buffers had no effect on the elution profile or the relative ratios of the pol- $\alpha$  multiple forms, precluding an involvement of serine proteases in non-specific generation of the activities during purification. Similar results have been reported for mouse mastocytoma (Bieri-Bonniot and Schurch, 1978), mouse myeloma (Chen *et al.*, 1979), and rat liver (Mechali *et al.*, 1980) pol- $\alpha$ s. Proteolysis by PMSF-resistant proteases cannot be ruled out at present, but the reproducibility of the ratios of the 3 forms in sonicated supernatants argues against their derivation from a single species through uncontrolled proteolysis during isolation. The 3 enzymatic activities therefore appear to be distinct components of the total embryonic chicken brain pol- $\alpha$  fraction rather than artifacts of preparation or resolution.

Attempts to classify the multiple forms as pol- $\alpha$ s by sedimentation velocity or isoelectric focusing experiments were thwarted by the remarkable instability of the resolved activities. The apparent preferences of the activities for Mg<sup>2+</sup> over Mn<sup>2+</sup> in activated DNA-supported reactions (table 2) implied that the 3 forms were pol- $\alpha$ s, but further substantiation was needed. Consequently, the sensitivities of the forms to known inhibitors of the eukaryotic DNA polymerases were assessed. Like the majority of previously characterized pol- $\alpha$ s, each of the 3 embryonic chicken brain

dramatically by this compound (figure 6a), which specifically inhibits pol- $\alpha$  (Onasiri *et al.*, 1978; Ikegami *et al.*, 1978; Pedrali-Noy and Spadari, 1979). Moreover, aphidicolin-induced inhibition of DNA synthesis was linearly competitive with dCTP (figure 6b), in accord with earlier characterizations of this inhibitor's mode of action (Oguro *et al.*, 1979; Sala *et al.*, 1980).

During the last decade a more refined understanding of bacteriophage DNA replication has evolved using a protein complex consisting of atleast 7 coded proteins (Alberts, 1984). All the accessory proteins of *Escherichia coli* holoenzyme pol III have recently been purified and cloned (Maki *et al.*, 1986). Out of 9 subunits it is the  $\alpha$ -subunit (140 kD) which is the catalytic subunit. However other subunits help in the highly processive replication of a prokaryotic DNA (e.g. Ori C. Plasmid). Our present attempts led us to resolve a complex that consists of DNA pol- $\alpha$  and primase activities. It is possible that this complex also contains a similar factor (NPF-1) which we have recently isolated from rat liver (Takada *et al.*, 1986) and it showed to stimulate the DNA chain initiation step (figure 8). Our results (Takada *et al.*, 1986a,b) suggest that this type of low molecular weight nonhistone proteins perhaps play important roles in the stabilization of the initiation complex (figure 8). Beside these accessory proteins very little is known about the chemical nature of the primases isolated from various animal tissues. Further characterization of this primase activity and its role in the embryonic development is under investigation.

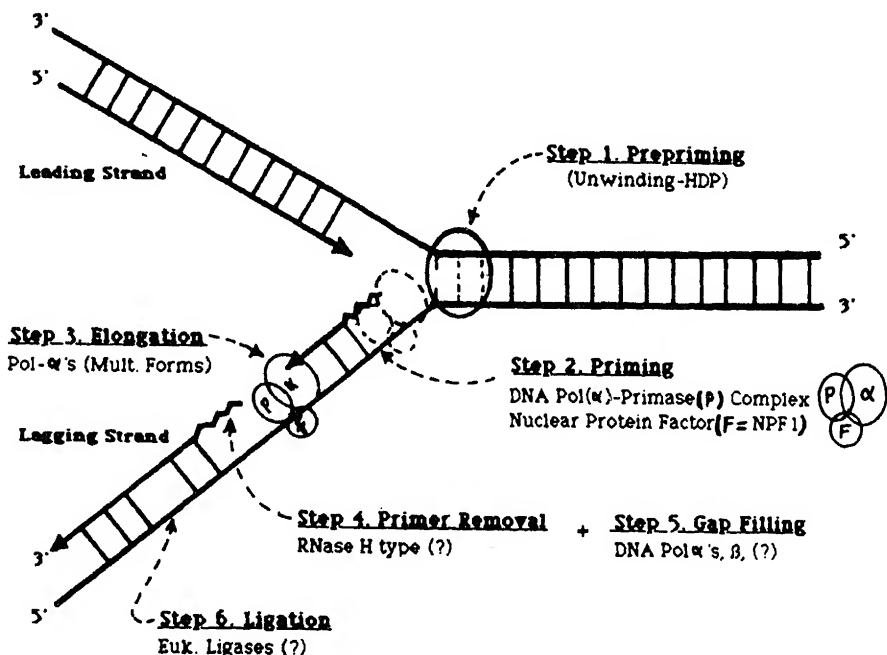


Figure 8. Proposed steps for discontinuous replication of eukaryotic DNA.

We wish to thank Dr. John Douros of the NIH for his help in securing for us a sample of aphidicolin and Dr. Shigeo Takada of the University of Notre Dame for the primase assay system used during this work. We thank Dr. Stephen Campion for his assistance in drawing some of the crucial figures. This work was supported by US Public Health Service Grants NS-18005 and CA-14764 to S.B.

## References

- Alberts, B. M. (1984) *Cold Spring Harb. Symp. Quant. Bio.*, **49**, 1.
- Bertazzoni, U., Scovassi, A. I. and Brun, G. M. (1977) *Eur. J. Biochem.*, **81**, 237.
- Bhattacharya, P., Simet, I. and Basu, S. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2683.
- Bieri-Bonniot, F. and Schurch, A. R. (1978) *FEBS Lett.*, **96**, 192.
- Bolden, A., Pedrali-Noy, G. and Weissbach, A. (1977) *J. Biol. Chem.*, **252**, 3351.
- Brakel, C. L. and Blumenthal, A. B. (1977) *Biochemistry*, **16**, 3137.
- Brakel, C. L. and Blumenthal, A. B. (1978) *Eur. J. Biochem.*, **88**, 351.
- Brun, G., Rougeon, F., Lauber, M. and Chapeville, F. (1974) *Eur. J. Biochem.*, **41**, 241.
- Burton, K. (1956) *Biochem. J.*, **62**, 315.
- Castroviejo, M., Tharaud, D., Tarrago-Litvak, L. and Litvak, S. (1979) *Biochem. J.*, **181**, 183.
- Chang, L. M. S. (1977) *J. Biol. Chem.* **252**, 1873.
- Chen, Y.-C., Bohn, E. W., Planck, S. R. and Wilson, S. H. (1979) *J. Biol. Chem.*, **254**, 11678.
- Duguet, M., Mechali, M. and Rossignol, J. M. (1978) *Anal. Biochem.*, **88**, 399.
- Filpula, D., Fisher, P. A. and Korn, D. (1982) *J. Biol. Chem.*, **257**, 2029.
- Grosse, F. and Krauss, G. (1985) *J. Biol. Chem.*, **260**, 1881.
- Hachmann, H. I. and Lezius, A. G. (1975) *Eur. J. Biochem.*, **50**, 357.
- Holmes, A. M., Cherialthundam, E., Bollum, F. J. and Chang, L. M. S. (1985) *J. Biol. Chem.*, **260**, 10840.
- Holmes, A. M., Hesselwood, I. P. and Johnston, I. R. (1974) *Eur. J. Biochem.*, **81**, 249.
- Hubscher, U. (1983) *Experientia*, **39**, 1.
- Hubscher, U., Kuenzle, C. C. and Spadari, S. (1977) *Eur. J. Biochem.*, **81**, 249.
- Hubscher, U., Spanos, A., Albert, W., Grummt, F. and Banks, G. R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6771.
- Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978) *Nature (London)*, **275**, 458.
- Kozu, T., Seno, T. and Yaguru, T. (1986) *Eur. J. Biochem.*, **157**, 251.
- Kaguni, L. S., DiFrancesco, R. A. and Lehman, I. R. (1984) *J. Biochem.*, **259**, 9314.
- Karkas, J. D., Marulies, L. and Chargaff, E. (1975) *J. Biol. Chem.*, **250**, 8657.
- Lamothe, P., Baril, B., Chi, A., Lee, L. and Baril, E. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4723.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Maki, H., Maki, S., Lasken, R. and Kornberg, A. (1986) *J. Cell Biochem., Suppl.* **10B**, 125.
- Masaki, S. and Yoshida, S. (1978) *Biochem. Biophys. Acta*, **521**, 74.
- Matsukage, A., Sivarajan, M. and Wilson, S. H. (1976) *Biochemistry*, **15**, 5305.
- Mechali, A., Abadiebat, J. and deRecondo, A. M. (1980) *J. Biol. Chem.*, **255**, 2114.
- Momparker, R. L., Rossi, M. and Labitan, A. (1973) *J. Biol. Chem.*, **248**, 285.
- Oguro, M., Suzuki-Hori, C., Nagano, H., Mano, Y. and Ikegami, S. (1979) *Eur. J. Biochem.* **97**, 603.
- Ohashi, M., Taguchi, T. and Ikegami, S. (1978) *Biochem. Biophys. Res. Commun.*, **82**, 1084.
- Ono, Y., Enomoto, T., Hanaoka, F. and Yamada, M. (1978) *Gann*, **69**, 207.
- Ono, Y., Enomoto, T. and Yamada, M. (1979) *Gann*, **70**, 527.
- Pedrali-Noy, G. and Spadari, S. (1979) *Biochem. Biophys. Res. Commun.*, **88**, 1194.
- Plevani, P., Foriani, M., Valsasini, P., Badaracco, G., Cherialthundam, E. and Chang, L. M. S. (1985) *J. Biol. Chem.*, **260**, 7102.
- Sala, F., Parisi, B., Burroni, D., Amileni, A. R., Pedrali-Noy, G. and Spadari, S. (1980) *FEBS Lett.*, **117**, 93.
- Shioda, M., Nelson, E. M., Bayne, M. L. and Benbow, R. M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7209.
- Simet, I. (1983) *Multiple forms of DNA polymerase- $\alpha$  from embryonic chicken brain*, Ph.D. Thesis, Univ. of

- Smith, J. and Soriano, L. (1977) *Nucleic Acids Res.* **4** 641.
- Spadari, S. and Weissbach, A. (1974) *J. Biol. Chem.*, **249**, 5809.
- Stavrianopoulos, J. G., Karkas, J. D. and Chargaff, E. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 2207.
- Stavrianopoulos, J. G., Karkas, J. D. and Chargaff, E. (1972a) *Proc. Natl. Acad. Sci. USA*, **69**, 1781.
- Stavrianopoulos, J. G., Karkas, J. D. and Chargaff, E. (1972b) *Proc. Natl. Acad. Sci. USA*, **69**, 2609.
- Takada, S., Torres-Rosado, A., Ray, S. and Basu, S. (1986a) *Proc. Fed. Am. Soc. Exp. Biol.*, **45**, 1779.
- Takada, S., Torres-Rosado, A., Ray, S. and Basu, S. (1986b) *Proc. Natl. Acad. Sci. USA*, **83**, 9348.
- Wang, T. S.-F., Hu, S.-Z. and Korn, D. (1986) *J. Biol. Chem.*, **259**, 1854.
- Wintersberger, E. (1978) *Eur. J. Biochem.*, **84**, 167.
- Yagura, T., Kozu, T. and Seno, T. (1982) *J. Biol. Chem.*, **257**, 1121.
- Yamaguchi, M., Matsukage, A. and Takahashi, T. (1980) *J. Biol. Chem.*, **255**, 7002.
- Yamaguchi, M., Tanabe, K., Takahashi, T. and Matsukage, A. (1982) *J. Biol. Chem.*, **257**, 4484.
- Yoshida, S., Kondo, T. and Ando, T. (1974) *Biochem. Biophys. Acta*, **353**, 463.
- Zimmerman, W., Chem, S. M., Holden, A. and Weissbach, A. (1980) *J. Biol. Chem.*, **255**, 11847.

# A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver

T. CHAKRABORTY, I. B. MAITI and B. B. BISWAS\*

Department of Biochemistry, Bose Institute, Calcutta 700 009, India

**Abstract.** Multiplicity of metallothionein and their genes in higher animals are documented extensively in recent literature. In contrast, chicken liver produced apparently a single form of metallothionein upon heavy metal exposure. This protein was purified by gel filtration and ion exchange chromatography and another technique based on heat treatment and acetone fractionation, followed by ion exchange chromatography. In adult uninduced chicken liver the presence of metallothionein was below the detection limit. But, like mammalian system, chicken liver was found to contain high amount of metallothionein at neonatal stage. This naturally occurring neonatal chicken hepatic metallothionein was purified and compared with the heavy metal induced adult hepatic metallothionein. The biochemical and immunobiological comparative analysis of adult and neonatal hepatic metallothionein showed identical characteristics. The neonatal metallothionein expressed naturally was a zinc metallothionein and unlike few other mammalian neonatal metallothionein did not contain any copper. Metallothionein was undetectable in unfertilized eggs, in early embryos, and in postnatal chicken, from 4 weeks after birth. The highest level of this naturally occurring neonatal metallothionein was found in 1–4 day old neonatal liver, which was about 1.5% of the total cytosolic protein. This is the first reported evidence for the presence of ontogenically modulated expression of metallothionein in avian system. Possible biological role of neonatal metallothionein and their cellular interactions has been discussed.

**Keywords.** Metallothionein; isometallothionein; neonatal; chicken liver; ontogenic-regulation; zinc; biological role.

## Introduction

Metallothionein (MT), a low molecular weight ( $M_r$ ), cystein-rich, inducible heavy metal binding protein is present in most of the eukaryotic organisms—from yeast to man (Kagi and Nordberg, 1979; Karin, 1985). A complete picture of its physiological role is not yet established but it appears to protect organisms against toxic heavy metals and may play certain role in the cellular zinc, and copper homeostasis (Foulkes, 1982; Hamer *et al.*, 1985). MT may be involved in defense against stress, infection or radiation damage (Oh *et al.*, 1978; Sobocinski and Canterbury, 1982; Thornalley and Vasak, 1985). The biosynthesis of MTs are increased, when organism or cultured cell lines are exposed to heavy metals such as zinc, copper, cadmium and mercury (Durnam and Palmiter, 1981). In addition to this, MT genes are induced by a plethora of chemical, physical or environmental stimuli which includes glucocorticoid hormones (Failla and Cousin, 1978; Karin and Herschman, 1980), interferon (Friedman and Stark, 1985), interleukin-1 (Karin *et al.*, 1985), bacterial LPS

\*To whom all correspondence should be addressed.

Abbreviations used: MT, Metallothionein;  $M_r$ , molecular weight; AP, acetone purified; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high performance liquid chromatography;

(Durnam *et al.*, 1984) hepatotoxic solvents like isopropanol or carbontetrachloride (Oh *et al.*, 1978; Swerdel and Cousin, 1984), UV, X-ray or  $\gamma$ -ray irradiation (Shiraishi *et al.*, 1983; Lieberman *et al.*, 1983) and starvation, strenuous exercise and other stresses (Oh *et al.*, 1978; Webb and Cain, 1982). The induction is regulated mainly at the level of transcription (Hager and Palmiter, 1981; Richards *et al.*, 1984; Searle *et al.*, 1985; Karin, 1984). A higher level of MT expression during early developmental stages of mammalian species has also been noted (Wong and Klassen, 1979; Piletz *et al.*, 1983; Anderson *et al.*, 1983; Andrews *et al.*, 1984). Thus, MT is a protein of potentially great biological importance; biochemical and genetic studies would contribute significantly in our understanding of its function and regulation.

In our laboratory, the biochemical and immunological comparative analysis of MTs isolated from different species and different tissues showed species specific difference in the ratio of isometallothioneins, difference in the electrophoretic migration as well as some tissue specific immunological variations (Chakraborty and Maiti, 1985). Attention is currently being paid on the multiplicity and microheterogeneity of isometallothioneins and their plausible physiological significance (Wilson *et al.*, 1982; Klauser *et al.*, 1983; Hunziker and Kagi, 1985). The chicken system was found to be unique among higher animals, as they produce apparently only a single form of MT under heavy metal induction. Under similar condition primates produce 6-7 different isometallothionein (Koizumi *et al.*, 1985). The ontogenic regulation of MT has been studied in different mammalian species. Since the early development of avian and mammalian species have certain marked differences, our interest was primarily focussed on the nature of MT in developing chicken liver. The present paper describes the purification, biochemical and immunological characterization of MT in developing chicken liver.

## Methods

White leghorn one day old chickens (average body weight 25 g), incubated fertilized eggs and developing neonatal chicks were obtained from State Poultry Farm, Tollygunge, (Govt. of West Bengal).

### Purification

For MT induction animals were injected with 0.5-2.0 mg  $\text{CdCl}_2/\text{kg}$  body weight and sacrificed after 18-24 h; livers were collected. Two different methods were used for purification of hepatic MTs. One was the usual and established methodology of gel filtration combined with ion-exchange chromatography (Chakraborty and Maiti, 1985). Another simple procedure for MT purification, based on its unusual heat stability and solvent fractionation behaviour was developed (Winge *et al.*, 1975). Tissue homogenate (1:5 w/v) in 10 mM Tris-HCl pH 8.6, 10 mM 2-mercaptoethanol was subjected to ultracentrifugation at 1,000,00  $g$  for 50 min. The clear supernatant avoiding lipid layer was taken and heat-treated at 85°C for 10 min, chilled on ice and centrifuged at 20,000  $g$  for 10 min. The clear supernatant (HTS)

on ice for 1 h, and centrifuged at 20,000 *g* for 30 min. This 60% acetone supernatant was made upto 80% (v/v) in acetone and kept at 0°C for 3 h and centrifuged. The 60–80% (v/v) acetone pellet was dissolved in 10 mM Tris-HCl pH 8.6 containing 5 mM 2-mercaptoethanol (or for spectrophotometric analysis, in H<sub>2</sub>O). This acetone purified fraction (AP) was found to be rich in MT. DEAE-sephadex chromatography of acetone purified fraction in 10 mM Tris-HCl pH 8.6 with a linear gradient of 10–300 mM of Tris-HCl pH 8.6 yielded a single homogeneous form of MT.

### *Gel electrophoresis*

Non-denaturing 7.5% polyacrylamide gel electrophoresis (PAGE) was done according to Davis (1964) in a slab gel. The gels were stained with 0.25% Coomassie Brilliant blue R-250 and destained as described earlier (Chakraborty and Maiti, 1985). For autoradiography, the gels were covered with Saran Wrap and dried in a LKB-Slab gel drier immediately after electrophoresis and dried gels were exposed to Kodak-X-ray films. Proteins were measured by the Bradford's dye binding assay (Bradford, 1976) using bovine serum albumin as standard.

### *Immunization and immunodouble diffusion assay*

Rabbits were immunized with purified chicken apometallothionein by method essentially same as that of Granger and Lazarides (1979) the details of which will be published elsewhere. The chicken MT antisera was tested by Ouchterloney immunodouble diffusion. The gels were stained and destained as for polyacrylamide gels. The results were confirmed with mouse MT antisera which also cross-reacts with chicken MT (Chakraborty and Maiti, 1985).

### *UV-Spectra*

UV absorption spectra of neonatal and Cd induced adult chicken hepatic MT were measured, using 15–30  $\mu$ g of purified MT in water, in a Shimadzu spectrophotometer. The change in pH was brought about by adding 1(N)HCl dropwise in the cuvette.

### *In vitro <sup>109</sup>Cd-labelling*

Naturally occurring neonatal MT was incubated with 10  $\mu$ Ci carrier free <sup>109</sup>CdCl<sub>2</sub> in 10 mM Tris-HCl pH 8.6 and 0.5 mM dithiothreitol (DTT) at 4°C for 12–16 h in a total volume of 100  $\mu$ l. The labelled proteins were separated from unbound free <sup>109</sup>CdCl<sub>2</sub> by passing it through a Sephadex G-25 column (0.8  $\times$  10 cm) equilibrated and eluted with 10 mM Tris-HCl pH 8.6, 0.5 mM DTT. The specificity of the labelling was judged by the autoradiography of 7.5% nondenaturing PAGE of the labelled products.



Sulphydryl groups were determined in purified MT fractions with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in a buffered solution of 6 M guanidine hydrochloride and 50 mM EDTA at pH 8.0 (Eldman, 1959), absorbance changes were monitored at 412 nm spectrophotometrically. The metals were analyzed using Varian Atomic Absorbance Spectrophotometer, using standard samples of zinc, cadmium and copper as references. Absorbance was monitored at 213.9 nm for zinc and 324.7 nm for copper in acetylene flame.

#### *High performance liquid chromatography (HPLC) and isoelectric focussing*

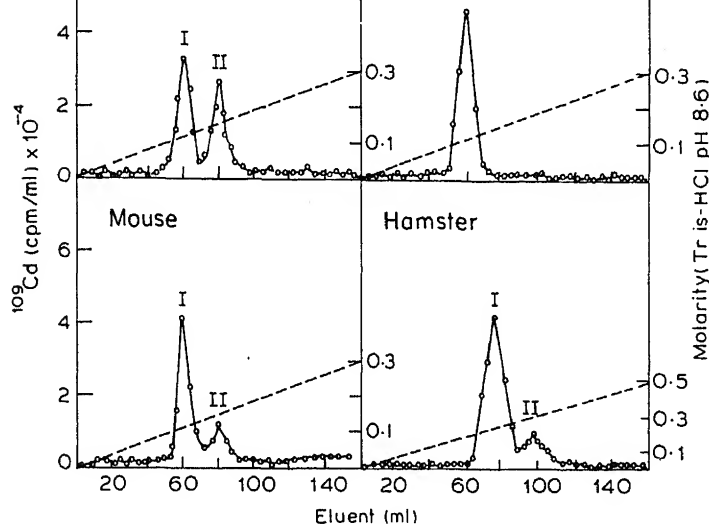
HPLC separation of MT was carried out in LKB apparatus using ultropac TSK G, 2000 SW column (7.5 × 300 mm) at 27°C. The elute was monitored at 220 or 206 nm. The flow rate was 0.1 ml/min, and the column was previously calibrated with known  $M_r$  protein markers (Suzuki, 1980).

The isoelectric focussing of purified MTs were carried out in 7.5% polyacrylamide gel rod, containing 2% carrier ampholine (Pharmalyte pI 3–10) in absence of any denaturing agent, and stained with Coomassie Brilliant blue G-250. The pIs were determined from parallelly run control gel rods.

## **Results**

### *Isolation and purification of MTs*

For large scale isolation from sources rich in MT the method based mainly on sephadex G-75 gel filtration followed by DEAE-ion exchanging chromatography gives good yield (Garvey *et al.*, 1982; Chakraborty and Maiti, 1985). For rapid isolation of MT from low amount of starting tissue material, we have adopted a methodology which gives good recovery in short time. This procedure is based upon the known heat stability and acetone precipitation behaviour of MT as described in methods section. We are using this method extensively in our study of the ontogenic regulation of MT synthesis in chicken. Figure 1 shows the typical DEAE-sephadex A-25 elution profile of MTs isolated from mouse, rat, golden hamster and chicken liver, after following identical induction and isolation procedure. The chickens, unlike most other higher animals produces apparently a single form of MT. Weser *et al.* (1973) have earlier isolated and characterized a MT from heavy metal induced chicken liver. They did not focussed on the isometallothionein pattern. The previous studies in our laboratory have clearly demonstrated that the ratio of isometallothioneins differs considerably from species to species and in some species like mouse and golden hamster one form is the major and another minor (Chakraborty and Maiti, 1985). Weser *et al.* (1973) isolated the MT after 14 days of the last heavy metal exposure, which might drastically affect the isometallothionein ratio (if they are really present). In our study, we followed the standard procedure for induction and



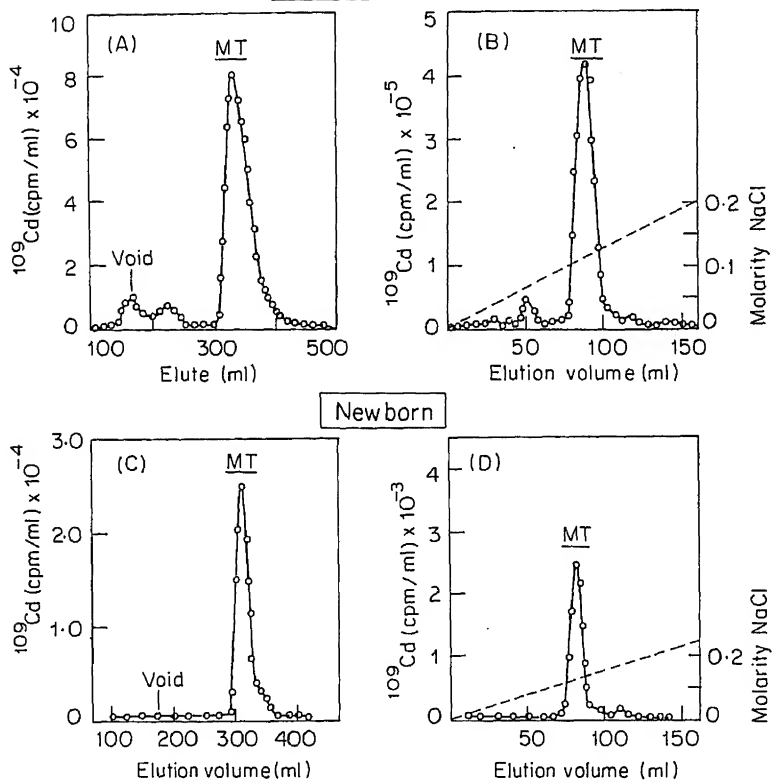
**Figure 1.** DEAE-sephadex A-25 column ( $6 \times 1$  cm) chromatography profiles of rat, mouse, golden hamster and chicken hepatic heavy metal induced MTs (sephadex G-75 pooled fraction, 3 mg). The proteins were eluted with a linear gradient 10–300 or 500 mM Tris-HCl pH 8.6 at a flow rate of 25 ml/h. The Cd-binding peaks denotes separated isoforms of MTs.

PLC hooked with gel permeation\* and isoelectric focussing. A single band of pI 4.1 was identified in isoelectric focussing gel.

#### *Purification of MT from newborn uninduced chicken liver*

The Cd-induced hepatic MT purified by Sephadex G-75 and DEAE-sephadex A-25 chromatography served as the standard for detection and identification of any similar protein in new born chick liver. Figure 2 (A and B) shows the elution pattern of cytosolic MT of Cd-administered chicken liver. Uninduced adult chicken liver subjected to identical isolation procedure did not show the presence of any MT like protein (data not shown). Three day old white leghorn chicken livers, when processed in identical manner, show the presence of a MT like protein. This protein can be easily *in vitro* labelled with  $^{109}\text{Cd}$ . The *in vitro*  $^{109}\text{Cd}$  labelled protein from neonatal livers on further fractionation in Sephadex G-75 and DEAE-sephadex A-25 chromatography behaved exactly like the hepatic induced MT (figure 2C and D). Neonatal hepatic MT was found to aggregate during lyophilization or prolonged storage. So, acetone fractionation procedure was adopted avoiding sephadex G-75 filtration step for neonatal MT purification.

The 60–80% acetone fraction was found to be rich in MT, comparable with Sephadex G-75 pooled protein. This scheme was found to be suitable for the quick isolation of MTs even from very low amount of starting material.



**Figure 2.** Purification of chicken adult Cd-treated and naturally occurring neonatal hepatic MTs. A and B show the sephadex G-75 column ( $90 \times 2.6$  cm) and DEAE-sephadex A-25 column ( $6 \times 1$  cm) elution profile of Cd-treated adult hepatic MTs. C and D show the neonatal *in vitro*  $^{109}\text{Cd}$ -labelled chicken hepatic proteins subjected to sephadex G-75 and DEAE-sephadex A-25 chromatography exactly like the adult Cd-induced samples.

### *Comparison of heavy metal induced and normal neonatal chicken liver MT*

An extensive comparative study of the heavy metal induced chicken liver MT with the normal neonatal hepatic MT was carried out. Result of this study is presented in table 1. In neonatal stage and also under heavy metal induction, the chicken was found to be capable of producing apparently a single form of MT. The UV-absorbance spectrum of the neonatal MT was very similar to all other animal MTs and the heavy metal induced MT of its own system (figure 3). The total absence of the aromatic amino acid residues is reflected in the very low absorbancy at 280 nm. The higher absorbancy at 250 nm is due to the metal-thiolate linkages. This 250 nm absorbance is abolished upon acidification due to the acid lability of the metal-thiolate bonds (Furey *et al.*, 1986; Low and Stillman, 1980). The electrophoretic migration ( $R_f$  value) of the neonatal and heavy metal induced MTs were identical on polyacrylamide gels in the presence (figure. 4B) or absence of sodium dodecyl sulphate (SDS) (table 1). The *in vitro*  $^{109}\text{Cd}$ -binding property of the neonatal MT and

$R_f$ value in 7.5% non denaturing PAGE	0.42	0.42
$M_r$ as determined by HPLC elution*	10 K	10 K
Metal content per mol of purified protein	6.8 g atom/mol $\pm$ 0.1	6.7 g atom/mol $\pm$ 0.1
No. of SH group/g atom of metal (ratio)		
per mol of protein	2.8	2.8
Absorbance 250 nm/280 nm ratio	High	High
Ability to <i>in vitro</i> bind $^{109}\text{Cd}$ .	+	+
Ability to cross-react with mouse MT-I antisera	+	+
Isoelectric point (pI)	4.1	4.1

\*Due to non-globular shape of MT the  $M_r$  determined on size exclusion or gel filtration principle gives higher than actual value. Moreover, at alkaline pH used, isometallothionein separate in this column (data not shown).

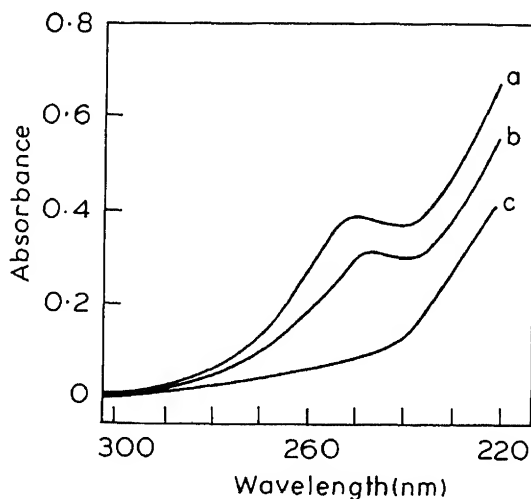
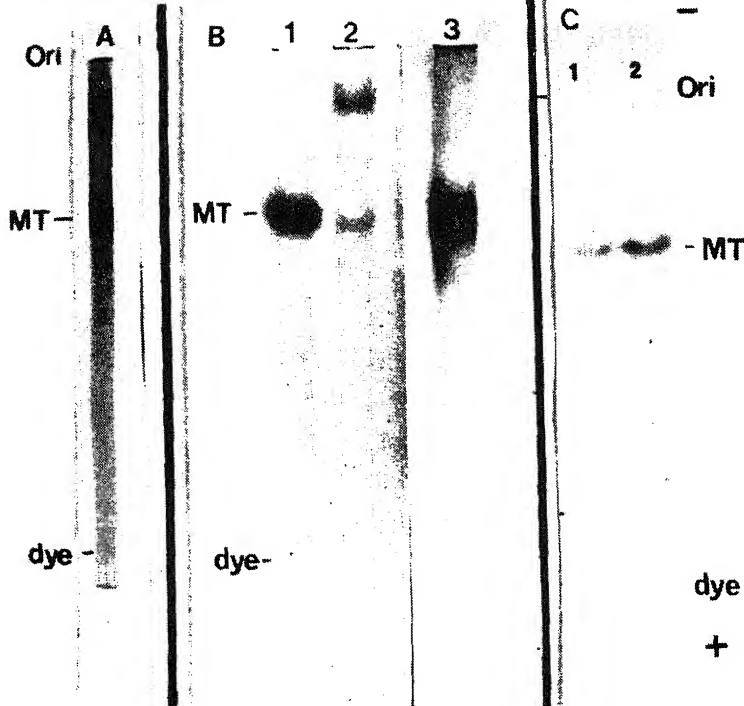


Figure 3. UV-absorption spectrum of neonatal chicken hepatic MT. The curve 'a' shows the spectrum at pH 7.0 of Cd-incubated neonatal MT, the curve 'b' shows the absorption spectra at pH 5.0 and the 'c' shows the UV-spectrum of the same sample at pH 2.0.

its co-migration with the *in vivo*  $^{109}\text{Cd}$  labelled Cd-induced chicken MT further confirm this view (figure 4C). Another critical parameter, which can distinguish the MT isoforms are the isoelectric-points, which was also found to be identical for both of the chicken MTs (table 1). The immunological identity of the proteins were evident, as they formed precipitating lines of complete identity in Ouchterlony's immunodiffusion test (figure 5). The cross-reactivity of both the mouse MT-I and chicken MT antisera with the chicken MT confirm our earlier observation that the mouse and chicken MT share immunological characteristics (Chakraborty and Maiti, 1985). Moreover, the HPLC elution patterns of both the MTs of chicken were same, and when a mixture of them were loaded on the column they are eluted as a single



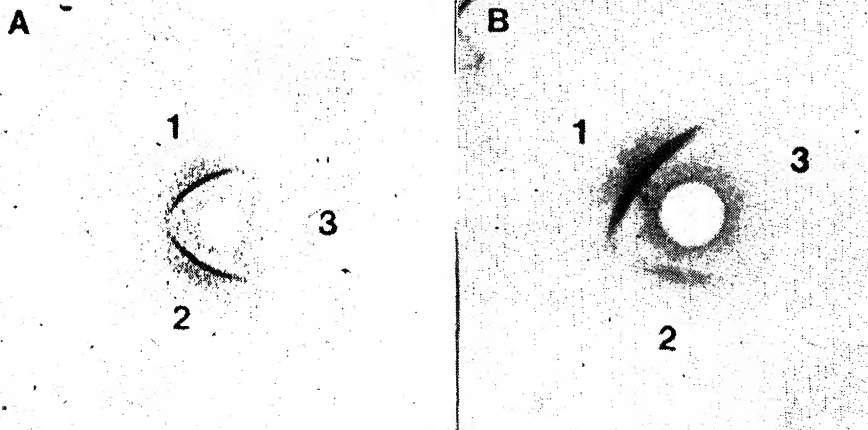
**Figure 4.** PAGE of chicken hepatic MT. A. Non-denaturing 7.5% PAGE of Cd-treated adult chicken hepatic MT of sephadex G-75 peak (30  $\mu$ g). The MT-band is associated with the  $^{109}\text{Cd}$ -radioactivity. B. Non-denaturing 7.5% PAGE of chicken hepatic proteins purified by the acetone fractionation scheme. Lane 1 and Lane 3 show the MTs purified from 1 day neonatal liver (20  $\mu$ g) and 20 day old embryonic liver (30  $\mu$ g) respectively. Lane 2 shows the sephadex G-75 MT peak of the neonatal chicken liver (25  $\mu$ g). C. The autoradiogram of the 7.5% PAGE separated *in vivo*  $^{109}\text{Cd}$ -induced adult chicken liver MT (Lane 1) and the *in vitro*  $^{109}\text{Cd}$ -labelled chicken neonatal MT (Lane 2).

The panel A and B are the Coomassie-blue stained polyacrylamide gels.

symmetric peak (table 1). The SH group and metal-ion ratio of both the MTs were found to be very comparable (table 1).

#### *Neonatal chicken MT is a Zn-MT*

Remarkable differences exist in the nature of the endogenous heavy metals associated with the naturally occurring neonatal hepatic MTs isolated from different animals. Fetal and neonatal mouse, chinese hamster and rat MT contain both zinc and copper in appreciable amount (Bakka and Webb, 1981). While in the syrian golden hamster and human fetal liver MT copper is the principal bound metal (Brady *et al.*, 1982). In contrast, the rabbit liver neonatal MT always contains zinc (Klaassen and Wong, 1982). In some species such as human fetus and infants, the zinc:copper ratio in the



**Figure 5.** Immunodouble diffusion assay of chicken MTs against mouse or chicken antimetallothionein. **A.** The central well contained rabbit antiserum prepared against chicken hepatic MT. The well 1 and 2 contained purified hepatic MT of Cd-treated adult and uninduced chicken neonatal MT, well 3 contained preimmune serum. **B.** The central well contained rabbit antiserum (immunoglobulin-G fraction) prepared against mouse hepatic MT-1. The well 1 and 3 contained chicken adult Cd-treated hepatic MT, chicken neonatal MT and uninduced adult chicken AP fraction of proteins.

chicken liver was found to be associated solely with zinc (table 1). There was no measurable copper in these samples. The metal and sulphhydryl content estimated from the same sample of MT showed that approximately all the metal binding sites of chick neonatal MT is saturated with zinc. The role of the neonatal MT in the metabolism and storage of zinc cannot be overlooked in these circumstances. The elevated MT in the neonatal liver has been postulated to serve as a transient store of essential heavy metals like zinc (Bell, 1979; Webb, 1979) and copper (Terao and Owen, 1977). In chicken the physiological involvement and interaction of neonatal MT must be sought in association with zinc and not with copper.

#### *Developmental regulation of MT*

In adult uninduced chicken liver MT is below our detection limit. This is reflected in the very low amount of protein in the 60–80% acetone purified fraction and the MT could not be detected even after *in vitro*  $^{109}\text{Cd}$  labelling. This is also reflected in immunological assay (figure 5B, well 3). This has earlier been observed in other adult animals not exposed to inducing agents (Winge and Rajagopalan, 1972). After establishing the presence of a naturally occurring MT in 3 day old neonatal chicken liver, detection of hepatic MT during chicken development was studied. The MT was not detectable in both unfertilized eggs and embryos of upto 2 weeks of development. Egg white, egg yolk and the embryo were tested by heat-treatment, acetone fractionation scheme followed by *in vitro*  $^{109}\text{Cd}$ -binding. Immunological assay also confirmed this result. This is consistent with the one earlier report, where only Cd

Datta, 1985). MT seems to appear during late maturation of the embryo and was abundant in 20 day embryo and 1–5 day old neonatal chicken liver. The concentration of protein (MT) declines steadily after the first week of birth and gradually disappears (below the detection limit) from one month old chicken liver but retains its capacity to be induced by heavy metals.

## Discussion

We have isolated MT from chicken liver after heavy metal exposure. A naturally occurring MT was also isolated from the neonatal chicken liver. The MT isolated under these two experimental conditions has been found to have one distinct property from those of the mammalian MTs. While all the mammalian MTs are known to be a mixture of at least two isoforms (isometallothioneins) which are separable by DEAE-anion exchange chromatography and in PAGE under non-denaturing condition (Koizumi *et al.*, 1985; Karin, 1985), this avian MT consists of a single isoform. Both the DEAE-chromatography and PAGE pattern of chicken MT conclusively establish this fact. As previous studies in this laboratory have shown that the ratio of isometallothioneins can vary widely, and in some species like mouse and golden hamster, the amount of one isometallothionein is predominant over the other, we have taken special care to see if any minor isometallothioneins are present together with the main chicken hepatic MT or not. The results obtained from  $^{109}\text{Cd}$ -labelling, HPLC, and isoelectric focussing ruled out this possibility. Many mammalian species have been shown to contain large amount of MT in their liver during prenatal and neonatal period (Ryden and Deutsch, 1978; Wong and Klassen, 1979; Bell, 1979, Charles-Shannon *et al.*, 1981). The mammalian hepatic MT at neonatal stage appears to be the mixture of two isometallothioneins like the adult induced ones (Webb and Cain, 1982). However, the validity of this phenomenon outside the mammalian domain has not been checked. We report here, the first evidence of such type of neonatal MT in avian species, containing only one form.

In the case of mammalian MT, whether the isoforms at neonatal stage are identical or different from the heavy metal induced MT isoforms have not been studied so far. We have done few comparative studies between the neonatal and heavy metal induced chicken MT. The biochemical and immunological properties like  $R_f$  value in denaturing and nondenaturing polyacrylamide gels, chromatographic elution pattern, HPLC analysis, isoelectric points and metal-sulphydryl ratios and immunological cross-reactivity suggest that these two MTs are identical. Whether there is any microheterogeneity within these isoforms (different species within a single form) can only be ascertained after amino acid sequence analysis which is now in progress.

Among all the vertebrates examined so far the multiple forms of MT have been detected under the experimental condition. However in the case of chicken it seems to be different. But, the number of MT isoforms and their genes are found to be variable among different vertebrates (Hunziker and Kagi, 1985; Klauser *et al.*, 1983; Karin, 1985). The complexity seems to be highest among the primates—where at least 6 different isoforms of MT and 12 genes including few pseudogenes, have been detected (Karin, 1985). Some of these mammalian isometallothioneins might have tissue specific expression and function (Chakraborty and Maiti, 1985; Schmidt and

the developmental regulation of MT biosynthesis in chicken so far studied by us seems to be similar to what has been found in mammalian system (Bakka and Webb, 1981; Piletz *et al.*, 1983; Andrews *et al.*, 1984). The full significance of this phenomenon is yet to be ascertained. Detailed study might reveal some biological involvement of MT. As the neonatal MT is found to be associated with zinc, the essential heavy metal closely associated with protein and nucleic acid biosynthesis, it is tempting to speculate that the function of this neonatal protein is to serve as a transient store of zinc which is toxic at high concentration in free form. The partitioning of zinc between MT and the protein and nucleic acid biosynthesizing machineries during development is one area to be investigated properly. The chicken MT antisera will be used to assay the detailed time course of the change in MT level from embryo to adult chickens. We are also engaged in studying this phenomenon at genetic level through MT-messenger RNA quantitation which will answer whether the elevation of MT is due to MT-gene switching during development. The importance of zinc as a principal regulatory element in gene expression as well as in the cellular adaptation to stress is increasingly being felt (Bruckman and Zondek, 1939; Ball, 1976; Brady, 1981; Fraker *et al.*, 1977; Good and Fernandes, 1979; Brady, 1982) and so association of neonatal chicken MT with zinc raises several interesting possibilities.

## Acknowledgement

Thanks are due to the Department of Science and Technology, New Delhi for the financial support.

## References

- Adamson, R. D., Piletz, J. E., Birren, B. W. and Herschman, H. R. (1983) *Eur. J. Biochem.*, **131**, 497.
- Andrews, G. K., Adamson, E. E. and Gedamu, L. (1984) *Dev. Biol.* **103**, 294.
- Bakka, A. and Webb, M. (1981) *Biochem. Pharmacol.*, **30**, 721.
- Ball, J. U. (1979) *Toxicol. Appl. Pharmacol.*, **50**, 101.
- Brady, F. O. (1981) *Life Sci.*, **28**, 1647.
- Brady, F. O., Webb, M. and Mason, R. (1982) in *Biological Roles of Metallothionein* (ed. E. C. Foulkes) (North Holland, Amsterdam: Elsevier) p. 77.
- Brady, F. O. (1982) *Trends Biol. Sci.*, **7**, 143.
- Bruckman, M. M. (1976) *Anal. Biochem.*, **72**, 248.
- Bruckman, G. and Zondek, S. G. (1939) *Biochem. J.*, **33**, 1845.
- Fraker, T. and Maiti, I. B. (1985) *Mol. Cell. Biochem.*, **68**, 41.
- Good, R. L., Shanon, V. L., Sasser, L. B., Burbunk, D. K. and Kelman, B. J. (1981) *Proc. Soc. Exp. Biol. Med.*, **168**, 56.
- Herschman, M. G. (1974) *Biochem. Biophys. Res. Commun.*, **61**, 920.
- Leucke, B. J. (1964) *Ann. N.Y. Acad. Sci.*, **121**, 248.
- Palmiter, D. M., Palmiter, R. D. (1981) *J. Biol. Chem.*, **256**, 5712.
- Palmiter, D. M., Hoffman, J. S., Quail, C. J., Benditt, E. P., Chen, H. Y., Brinster, R. L. and Palmiter, R. D. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1053.
- Palmiter, G. L. (1959) *Arch. Biochem. Biophys.*, **82**, 70.
- Palmiter, M. L. and Cousin, R. J. (1978) *Biochim. Biophys. Acta.*, **543**, 293.
- Foulkes, E. C. (ed.) (1982) *Biological Roles of Metallothionein*, (North-Holland, Amsterdam: Elsevier)
- Fraker, R. J., Haas, S. and Leucke, R. W. (1977) *J. Nutr.*, **107**, 1889.
- Goodman, R. L. and Stark, G. R. (1985) *Nature (London)*, **314**, 637.
- Gray, W. F., Robbins, A. H., Clancy, L. L., Winge, D. R., Wong, B. C. and Stout, C. D. (1986) *Science*,



- Garvey, J. S., Vander Mallie, R. J. and Chang, C. C. (1982) *Methods Enzymol.*, **84**, 121.
- Good, R. R. and Fernades, G. (1979) *Clin. Bull.*, **9**, 3.
- Granger, B. L. and Lazarides, E. (1979) *Cell*, **18**, 1053.
- Hager, L. J. and Palmiter, R. D. (1981) *Nature (London)*, **291**, 340.
- Hamer, D. H., Thiels, D. J. and Lemont, J. E. (1985) *Science*, **228**, 685.
- Hunziker, P. E. and Kagi, J. H. R. (1985) *Biochem. J.*, **231**, 375.
- Kagi, J. H. R., Nordberg, M. (eds) (1979) *Metallothionein*, (Basel: Birkhauser, Verlag).
- Karin, M. (1985) *Cell*, **41**, 9.
- Karin, M. and Herschman, H. R. (1980) *J. Cell. Physiol.*, **103**, 35.
- Karin, M., Imbra, J. R., Hague, A. and Qong, G. (1985) *Mol. Cell. Biol.*, **5**, 2866.
- Klaassen, C. D. and Wong, K. L. (1982) in *Biological roles of Metallothionein* (ed. E. C. Foulkes) (North-Holland: Elsevier) p. 113.
- Klauser, S., Kagi, J. H. R. and Wilson, K. L. (1983) *Biochem. J.*, **209**, 71.
- Koizumi, S., Otaki, N. and Kimura, M. (1985) *J. Biol. Chem.*, **260**, 3672.
- Lieberman, M. W., Beach, L. R. and Palmiter, R. D. (1983) *Cell*, **35**, 207.
- Low, A. Y. C. and Stillmann, M. J. (1980) *Biochem. Biophys. Res. Commun.*, **94**, 138.
- Oh, S. H., Deagen, J. T., Whanger, D. D. and Weswig, P. H. (1978) *Am. J. Physiol.*, **234**, E-282.
- Piletz, J. E., Anderson, R. D., Birren, B. W. and Herschman, H. R. (1983) *Eur. J. Biochem.*, **131**, 489.
- Prasad, A. R. K. and Datta, K. (1983) *Biochem. Int.*, **7**, 677.
- Richards, R. I., Hegue, A. and Karin, M. (1984) *Cell*, **37**, 263.
- Ryden, L. and Deutsch, H. F. (1978) *J. Biol. Chem.*, **253**, 519.
- Schmidt, C. J. and Hamer, D. H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3346.
- Searle, P. F., Davison, B. L., Stunt, G. W., Wilkie, T. M., Norstedt, G. and Palmiter, R. D. (1984) *Mol. Cell. Biol.*, **4**, 1221.
- Shiraishi, N., Aono, K. and Utsumi, K. (1983) *Radiat. Res.*, **95**, 298.
- Sobocinski, P. Z. and Canterbury, W. T. (1982) *Ann. N.Y. Acad. Sci.*, **389**, 354.
- Suzuki, K. T. (1980) *Anal. Biochem.*, **102**, 31.
- Swerdel, M. R. and Cousin, R. J. (1984) *Proc. Soc. Exp. Biol. Med.*, **175**, 522.
- Terao, T. and Owen, C. A. (1977) *Am. J. Physiol.*, **232**, E-172.
- Thornally, P. J. and Vasak, M. (1985) *Biochim. Biophys. Acta.*, **827**, 36.
- Vallee, B. L. (1976) in *Cancer Enzymology* (eds J. Schultz and F. Abnad) (New York: Academic Press) p. 1.
- Webb, M. (ed) (1979) *The Chemistry, Biochemistry and Biology of Cadmium* (New York: Elsevier-North Holland).
- Webb, M. and Cain, K. (1982) *Biochem. Pharmacol.*, **31**, 137.
- Weser, U., Rupp, H., Donay, F., Linnemann, F., Voelter, W., Voetsch, W. and Jung, G. (1973) *Eur. J. Biochem.*, **39**, 127.
- Wilson, K. J., Weiringen, V., Klauser, E., Berchtold, M. W. and Hughes, G. J. (1982) *J. Chromatogr.*, **237**, 407.
- Winge, D. R., Premakumar, R. and Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys.*, **170**, 242.
- Winge, D. R. and Rajagopalan, K. V. (1972) *Arch. Biochem. Biophys.*, **153**, 755.
- Wong, K. L. and Klaassen, C. D. (1979) *J. Biol. Chem.*, **254**, 12399.

# Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat

NANDINI NAIR and C. K. RAMAKRISHNA KURUP\*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

**Abstract.** It is shown for the first time that the content of ubiquinone of liver increases (2.5 fold) on dietary administration of the widely-used industrial plasticizer diethylhexyl phthalate to the rat. The increase is localized almost entirely in mitochondria in which the concentration of the quinone per mg protein is 1.7 times the control. Incorporation of the radioactive precursor (acetate) reveals that the biosynthesis of ubiquinone is increased in the livers of plasticizer-administered animals. The rate of degradation is not altered.

**Keywords.** Diethylhexyl phthalate; liver; ubiquinone; increase; synthesis.

## Introduction

The plasticizer, di(2-ethylhexyl) phthalate (DEHP, also called dioctyl phthalate), is a major constituent of flexible plastics used in the packaging of food stuffs and in the manufacture of medical devices. Since it is not covalently bound, the phthalate ester may easily leach out of the plastic. Thus, the compound has been detected in stored blood as well as in the tissues of patients receiving blood transfusion. Because of this and its occurrence ubiquitously in the soil, plant and stored food, the toxicity of the plasticizer has been investigated in detail (Thomas *et al.*, 1978). Administration of the plasticizer to experimental animals produces profound biochemical changes in the liver. Most of these changes resemble those produced by the antihypercholesterolemic drug clofibrate. Recently, we have investigated the serum cholesterol-lowering property of DEHP first reported by Reddy *et al.* (1976) and have shown that the compound regulates the concentration of cholesterol in circulation both by inhibition of synthesis and by stimulation of degradation (Nair and Kurup, 1986).

Based on the observation that oral administration of ubiquinone effectively increases its concentration in the liver and also inhibits the synthesis of cholesterol *in vivo*, the hypothesis has been advanced that hypocholesterolemic agents exert their physiological action *via* ubiquinone (Krishnaiah, *et al.*, 1967a). Support for this view was provided by the fact that the lipid quinone branches from the biosynthetic pathway of cholesterol (Ramasarma, 1972) and the observation that administration of clofibrate increases the concentration of ubiquinone in liver (Krishnaiah *et al.*, 1967b; Philips *et al.*, 1968; Krishnaiah and Ramasarma, 1970; Kurup *et al.*, 1970; Krishnakantha and Kurup, 1974). Further, it was reported from our laboratory that the azodye 2-methyl-4-dimethyl-aminóbenzene depressed the concentration of cholesterol in circulation and raised the content of ubiquinone in the livers of rats (Saikumar and Kurup, 1984). It was of interest to see whether the correlation between serum cholesterol level and hepatic ubiquinone content holds good in the

case of DEHP also. The data presented in this paper reveal for the first time that administration of the plasticizer to the rat increases the concentration of ubiquinone in the liver and that the increase is achieved by a stimulation of the biosynthetic rate.

## Materials and methods

### *Animals*

Male albino rats (130–150 g) fed with a commercial Hind Lever diet (vegetable based; carbohydrate 53%, protein 25%, minerals 9%, fat 5% and fibre 4%) and kept in a room with artificial lighting (from 9-00 a.m. to 5-00 p.m.) were used in the experiments reported here. The phthalate ester was fed mixed in the diet powder (2% w/w) for the time period indicated.

### *Subcellular fractionation*

Animals were killed by cervical dislocation and hepatic mitochondria isolated by differential centrifugation (Kurup *et al.*, 1970). The particles were washed once with 0.25 M sucrose and used for estimation of ubiquinone. The microsomal fraction was sedimented by centrifugation at 39,000 *g* for 1 h.

### *Estimation of ubiquinone*

The content of ubiquinone in liver tissue and subcellular fractions was determined by spectrophotometry after saponification, extraction and chromatography on deactivated alumina (Joshi *et al.*, 1963). The concentration of the quinone was calculated from the decrease in absorbance at 340 nm of an ethanolic solution on reduction with  $\text{NaBH}_4$  (Ramasarma, 1968). The content was also estimated by high performance liquid chromatography in a LKB unicord S II system using a Lichrosorb RP18 column ( $4 \times 250$  nm) and ethanol as the eluent (flow rate 1 ml/min). Concentration was calculated from peak area using standard ubiquinone-9 (Abe *et al.*, 1981). In order that 'cyclic' variations (Edwin *et al.*, 1962) may not vitiate the results, an equal number of control and experimental animals were killed in the morning.

### *Turnover of ubiquinone*

The rate of synthesis of ubiquinone was followed by determining the incorporation of  $[1-^{14}\text{C}]$ -acetate. The precursor was injected intra-peritoneally and the animals were killed 30 min later. To determine the decay rates of ubiquinone, rats were injected with the precursor and killed 1, 2 and 4 days later. The livers were processed for the isolation and estimation of ubiquinone.

Protein was estimated by the biuret method, deoxycholate being used for solubilization (Gornall *et al.*, 1949). Bovine serum albumin was used as standard. Samples for determination of radioactivity were dissolved in benzene (0.5 ml), added to vials containing 10 ml of 2,5-diphenyloxazole (0.5% w/v) in toluene and counted in a LKB Rack-Beta II Liquid Scintillation Counter. The background was deducted from all

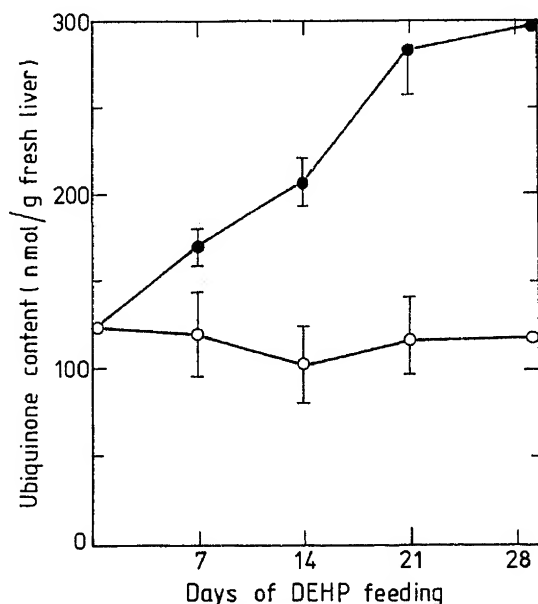
re, Bombay. All biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Other chemicals used were of the purest grades available. Solutions were prepared in water double-distilled in all-quartz apparatus and adjusted to the desired pH before use.

## Results

### Ubiquinone content of liver

Administration of the plasticizer results in an increase in the concentration of ubiquinone in the liver. Significant increase (30%;  $P < 0.05$ ) is observed in a week's time. In about 3 weeks the maximum increase (about 150%) is obtained (figure 1). The values for control animals agree closely with those reported in the literature (Masaruma, 1985a). It may be pointed out that the increase obtained is of the same order (2–3-fold) as has been observed on administration of clofibrate (Krishnakantha and Kurup, 1974).

It may be pertinent to mention in this context that the diet contained  $8.8 \pm 1.4$  nmol,



**Figure 1.** Effect of administration of diethylhexyl phthalate (DEHP) to the rat on the concentration of ubiquinone in liver. The concentration of ubiquinone (nmol) per g of liver in control (○) and DEHP-fed (2% w/w) in the diet (●) animals are given. Some typical values of standard deviation are also indicated. Experimental details are given in the 'materials and methods' section.

of ubiquinone/g. The average dietary consumption of ubiquinone in control and DEHP-fed animals was 146 and 149 nmol/rat per day respectively.

### Ubiquinone synthesis

In order to gain an insight into the mechanism of increase of hepatic ubiquinone, the incorporation of acetate into the lipid quinone was studied. The results presented in table 1 reveal that administration of the plasticizer stimulates the synthesis of the quinone in liver. The incorporation of the precursor into the non-saponifiable lipid fraction per g of liver is decreased by almost 50% in DEHP-fed animals. This is consistent with our observation that in these animals both the specific activity of 3'-hydroxy 3-methyl glutaryl (HMGCoA) reductase (Mevolonate: NADP oxidoreductase (acylating CoA), EC 1.1.1.34) and the incorporation of acetate into hepatic cholesterol are inhibited by 50% (Nair and Kurup, 1986). Incorporation of the precursor into ubiquinone which is only a small fraction ( $4 \pm 1\%$ ) of the total incorporation into the non-saponifiable lipids of whole liver in control animals, increases more than 5-fold ( $21 \pm 8\%$ ) in the plasticizer-fed ones. The specific radioactivity of ubiquinone also is significantly ( $P < 0.01$ ) higher in the phthalate-ester-fed animals ( $2572 \pm 332$  counts/min/ $\mu$ mol) than that in the control ones ( $1329 \pm 207$  counts/min/ $\mu$ mol).

**Table 1.** Effect of administration of diethylhexyl phthalate on the synthesis of ubiquinone in rat liver.

Lipid fraction	Counts/min per g liver	
	Control	Plasticizer-fed
Non-saponifiable	$5883 \pm 800$ (100)	$3766 \pm 448^*$ (64)
Ubiquinone	$221 \pm 8$ (4 $\pm$ 1)	$839 \pm 187^*$ (14 $\pm$ 3)

Rats were injected with [ $1\text{-}^{14}\text{C}$ ]-acetate and killed 30 min later. The plasticizer was given in the diet (2% w/w) for 30 days. The values are the mean  $\pm$  S.D. of 4 independent determinations (animals). The values in parentheses indicate the incorporation taking total incorporation into the non-saponifiable lipids of control animals as 100, \* $P < 0.01$  Control vs experimental.

### Ubiquinone turnover

Determination of the rate of degradation of the quinone was done by measurement of the decay of radioactivity (Waterlow *et al.*, 1978). The rate constant  $K_d$  was calculated from the equation

$$\ln \frac{A_0}{A_t} = K_d \cdot t,$$

of the plot of  $\ln A_t$  against  $t$  yielded  $K_d$ . By definition, half-life is given by the equation

$$t_{\frac{1}{2}} = \frac{0.693}{K_d}.$$

The half-life of ubiquinone calculated in this manner for control ( $2.6 \pm 0.2$  days) and plasticizer-fed (30 days) animals ( $2.5 \pm 0.4$  days) is the same. These values agree well with the half life (2–3 days) calculated from the data of Joshi and Ramasarma (1966).

### *Mitochondrial ubiquinone*

A major portion of the ubiquinone in the cell is localized in the mitochondrial fraction. Administration of the plasticizer is known to cause enlargement of the liver (Warren *et al.*, 1982). In order to see whether the increase in the content of ubiquinone in liver reflects only the increase in the population of mitochondria or not, the concentration of the quinone in the mitochondrial fraction was determined. The data presented in table 2 show that the content of ubiquinone in the mitochondrial membrane (per mg protein) increases by more than 70% ( $P < 0.01$ ). Our values for control animals agree well with those reported in the literature (Sastry *et al.*, 1961; Krishnakantha and Kurup, 1974). Quite in agreement with previous reports (Sastry *et al.*, 1961; Ramasarma, 1985a), the mitochondrial fraction accounts for about half of the total cellular ubiquinone (table 2). The proportion increases by 78% in plasticizer-fed animals. It may be stated that the microsomal fraction did not show any significant difference in the content of ubiquinone between control ( $30 \pm 6$  nmol/100 mg protein) and phthalate ester-fed ( $23 \pm 6$  nmol/100 mg protein) animals.

**Table 2.** Effect of administration of diethylhexyl-phthalate on the content of ubiquinone in rat liver mitochondria.

Mitochondrial ubiquinone	Control	Plasticizer-fed (nmol)
Per 100 mg protein	$162 \pm 19$	$277 \pm 24^*$
Per g liver	$63 \pm 7$ (53)	$223 \pm 16^*$ (75)
Per whole liver	$386 \pm 41$ (53 $\pm$ 6)	$2325 \pm 278^*$ (78 $\pm$ 8)

Rats were administered with DEHP in the diet (2% w/w) for 30 days. The values in parentheses represent the content of ubiquinone in the mitochondrial fraction taking the corresponding liver ubiquinone as 100. The values are the mean  $\pm$  S.D. of 4 independent determinations (animals). \* $P < 0.01$  Control *vs* experimental.

The data presented in table 2 were obtained by determining the concentration of ubiquinone by the spectrophotometric method. To confirm that the large increase of ubiquinone in the mitochondrial fraction was not an artifact of interference by an ultraviolet-absorbing reducible substance produced by the ester, the quinone content

matography. The values obtained by the latter method were  $92 \pm 6\%$  of the values by the spectrophotometric method in control and  $120 \pm 6\%$  in plasticizer-fed animals.

## Discussion

The data presented here which form the first report on the ability of DEHP to increase the concentration of hepatic ubiquinone *in vivo*, is consistent with the hypothesis (Ramasarma, 1967) that the modulation of HMGCoA reductase activity achieved by hypocholesterolemic agents is mediated by ubiquinone. We have observed that administration of plasticizer causes a substantial (50%) decrease in the specific activity of the enzyme in hepatic microsomes (Nair and Kurup, 1986).

It may appear paradoxical that when the activity of the rate-limiting enzyme is inhibited and the pool size of mevalonate is small, the synthesis of ubiquinone is enhanced. Similar results have been reported in cholesterol feeding also (Krishnaiah *et al.*, 1967a). It is generally believed that in rat liver the availability of mevalonate and not that of the ring precursor limits the biosynthetic rate of the quinone (Ramasarma, 1985b). Enhanced biosynthesis of ubiquinone from acetate is possible even when the rate-limiting enzyme is inhibited because the relative incorporation of acetate into ubiquinone is insignificantly small (about 4% of the non-saponifiable fraction) while the incorporation into cholesterol (90%) is very large. Moreover, the possible existence of a regulatory step in the conversion of squalene to lanosterol (Cenedella, 1980; Volpe and Obert, 1982) would favour channeling of the precursor into the quinone biosynthetic pathway.

Increasing the content of hepatic ubiquinone is a property which the plasticizer shares with clofibrate. However the intra-cellular distribution of ubiquinone in the two cases is not similar. In clofibrate-fed animals the concentration of ubiquinone in the mitochondrial membrane (per mg protein) is not increased. The increase is substantial on the otherhand, in the nuclear fraction (Krishnakantha and Kurup, 1974). In the case of the plasticizer, the increase in ubiquinone appears to be accounted for exclusively by the mitochondrial fraction.

## References

- Abe, K., Kattayama, K., Ikenoya, S., Takada, M., Yuzuriha, T., Hanamura, K., Nakamura, T., Yusuda, K., Yoshida, S., Watson, B. and Kogure, K. (1981) in *Biochemical and Clinical Aspects of Coenzyme Q* (eds K. Folkers and Y. Yamamura) (Amsterdam: Elsevier) Vol. 3, p. 53.
- Cenedella, R. J. (1980) *Biochem. Pharmacol.*, **29**, 2751.
- Edwin, E. E., Bunyan, J., Green, J. and Diplock, A. T. (1962) *Br. J. Nutr.*, **16**, 135.
- Ganning, A. E., Brunk, U. and Dallner, G. (1983) *Biochim. Biophys. Acta.*, **763**, 72.
- Gornall, A. G., Bardawill, G. J. and David, M. M. (1949) *J. Biol. Chem.*, **177**, 751.
- Joshi, V. C., Jayaraman, J. and Ramasarma, T. (1963) *Indian J. Exp. Biol.*, **1**, 113.
- Joshi, V. C. and Ramasarma, T. (1966) *Biochim. Biophys. Acta.*, **115**, 294.
- Krishnaiah, K. V., Joshi, V. C. and Ramasarma, T. (1967a) *Arch. Biochem. Biophys.*, **121**, 147.
- Krishnaiah, K. V., Inamdar, A. R. and Ramasarma, T. (1967b) *Biochem. Biophys. Res. Commun.*, **27**, 474.
- Krishnaiah, K. V. and Ramasarma, T. (1970) *Biochem. J.*, **116**, 321.
- Krishnakantha, T. P. and Kurup, C. K. R. (1974) *Indian J. Biochem. Biophys.*, **11**, 51.
- Kurup, C. K. R., Aithal, H. N. and Ramasarma, T. (1970) *Biochem. J.*, **116**, 773.
- Nair, N. and Kurup, C. K. R. (1986) *Biochem. Pharmacol.*, **35**, 3441.
- Philips, W. F. J., Lakshmanan, M. R. and Brien, R. I. (1968) *Can. J. Physiol. Pharmacol.*, **46**, 81.

- Ramasarma, T. (1968) *Adv. Lipid Res.*, **6**, 107.
- Ramasarma, T. (1972) *Curr. Topics Cell. Reg.*, **6**, 169
- Ramasarma, T. (1985a) in *Coenzyme Q* (ed. G. Lenaz) (Chichester: John Wiley) p. 131.
- Ramasarma, T. (1985b) in *Coenzyme Q* (ed. G. Lenaz) (Chichester: John Wiley) p. 67.
- Reddy, J. K., Moody, D. E., Azarnoff, D. L. and Rao, M. S. (1976) *Life Sci.*, **18**, 941.
- Saikumar, P. and Kurup, C. K. R. (1984) *Biochem. J.*, **224**, 955.
- Sastry, P. S., Jayaraman, J. and Ramasarma, T. (1961) *Nature (London)*, **189**, 557.
- Thomas, J. A., Darby, T. D., Wallin, R. F., Garvin, P. J. and Martis, L. (1978) *Toxicol. Appl. Pharmacol.*, **45**, 1.
- Volpe, J. J. and Obert, K. A. (1982) *J. Neurochem.*, **38**, 931.
- Warren, J. R., Lalwani, N. D. and Reddy, J. K. (1982) *Environ. Health Perspect.*, **45**, 35.
- Waterlow, J. C., Garlick, P. J. and Millward, D. J. (1978) *Protein Turnover in Mammalian Tissues and in the whole Body* (Amsterdam: North Holland).





# Effect of mitogenic factors extracted from fetal lung fibroblasts on the *in vitro* growth of melanocytes obtained from normal and vitiligo subjects

MANOJ MOJAMDAR, NEELU PURI and ABBURI RAMAIAH\*

Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110 029, India

**Abstract.** The effects of growth factors extracted from a newly established fetal lung fibroblast cell line (PMR-GF) on the melanocytes cultured from the perilesional and depigmented skins of vitiligo subjects and from normal healthy donors have been investigated. Melanocytes from normal subjects grown in the presence of 10 ng/ml of 12-O-tetradecanoyl phorbol 13-acetate and  $10^{-11}$  M cholera toxin grew exponentially immediately after seeding the epidermal cell suspensions. Exogenous addition of PMR-GF to these cells enhanced their growth rates. The perilesional skin melanocytes of vitiligo subjects in most cases did not manifest any growth when cultured in the presence of 12-O-tetradecanoyl phorbol 13-acetate and cholera toxin. PMR-GF induced a brief burst of growth in these cells after a lag of 15 days. Vitiligo lesions gave rise to a few unpigmented dendritic cells that did not manifest any growth in the presence or absence of PMR-GF. Morphologically the perilesional skin melanocytes of most vitiligo subjects, when cultured in 12-O-tetradecanoyl phorbol 13-acetate and cholera toxin, appeared to be larger and hyper-melanotic as compared to those of normal individuals. In the presence of PMR-GF these melanocytes appeared to be normal in size and less hyper-melanotic. Our results indicate that the melanocytes from vitiligo subjects are defective and thus the basic defect in vitiligo could be with the melanocytes themselves.

**Keywords.** Melanocyte culture; growth factors; human vitiligo.

## Introduction

Vitiligo, a disease characterized by patchy depigmentation of the skin, is believed to affect 1% of the world population (Lerner, 1959). In India, however, its incidence is believed to be 3–4% (Robert, 1941). In the perilesional skin surrounding the vitiligo macule, the melanocytes were found to be reduced in numbers and morphologically abnormal, as though they had been fixed in the  $G_2$  phase of the cell cycle (Lerner and Nordlund, 1978). Different theories as to the cause of this disease, propose different mechanisms for the destruction of melanocytes in these patients.

We have recently demonstrated by sensitive biochemical techniques, the presence of tyrosinase the melanocyte marker enzyme in the vitiligo lesions (Hussain *et al.*, 1982). The levels of tyrosinase in these lesions were 4–37% of the corresponding normal skins of the same subjects, suggesting that the melanocytes in these macules may have become quiescent (figure 1). We have therefore, initiated studies on the cultures of melanocytes from normal and vitiligo subjects (Puri *et al.*, 1987). We report here that fetal lung fibroblast extracts that promote the growth of melanocytes obtained from normal donors, do not sustain the growth of melanocytes

\*To whom all correspondence should be addressed.

Abbreviations used: MEM, Minimum essential medium; TPA, 12-O-tetradecanoyl 13-acetate; CT, Cholera toxin; PBS, Phosphate buffered saline; DAM-Smyth, delayed amelanotic-Smyth.

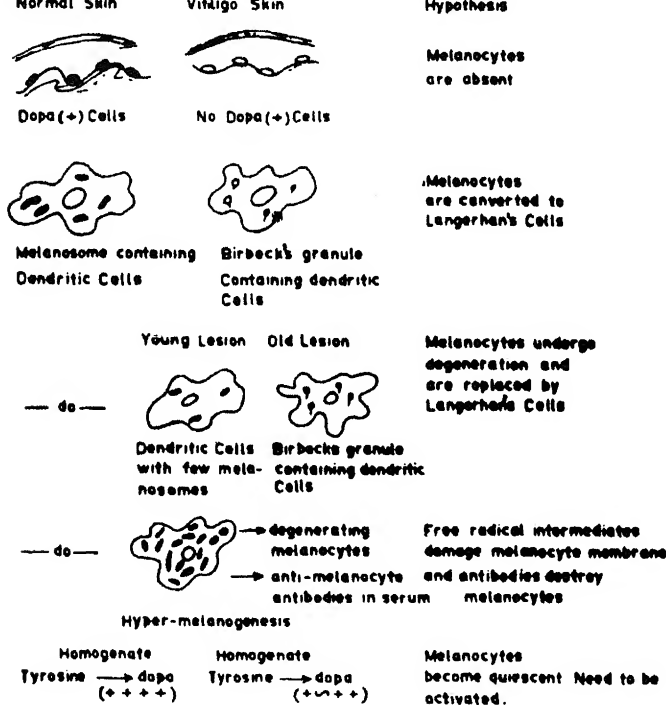


Figure 1. Progressive changes in the etiopathological concepts of vitiligo. Detailed analysis of these theories can be seen in Lerner (1959), Lerner and Nordlund (1978), Mishima *et al.* (1972) and Ramaiah (1985).

of the perilesional skins of vitiligo subjects and has no effect on the unpigmented dendritic cells that the white lesions give rise to. Based on these results we conclude that the basic defect in vitiligo lies with the melanocytes themselves.

## Materials and methods

### *Patients and culture techniques*

Vitiligo vulgaris patients appearing at our hospital pigmentation clinic were carefully screened. Only those patients who had not taken any medicine for at least a year and had no other concurrent ailments were chosen for the present study (table 1). Intermediate thickness split-skin biopsies were obtained under local anesthesia (Lidocaine 1%) from clear milky white patches and the perilesional hypo- or hyperpigmented borders. The biopsies were immediately washed 6–7 times with Eagles minimum essential medium (MEM) containing antibiotics (penicillin 1000 µg/ml, streptomycin 1000 µg/ml and fungizone 0.25 µg/ml) and were kept in 0.25% trypsin for 13 h at 4°C. The epidermis was then separated from the dermis and shaken vigorously in 0.05% trypsin solution containing 0.02% EDTA. The epidermal cell suspensions were then seeded into Linbro (Flow laboratories, USA) 24-well plates in

**Table 1.** Case histories of vitiligo subjects.

Initials	Age	Sex	Duration of disease	Site of biopsy
A.K.	18	M	6	Thigh
S.L.	24	M	7	Arm
I.K.	25	F	20	Trunk
R.N.A.	32	M	10	Trunk
R.O.G.	45	M	30	Trunk
B.L.M.	42	M	20	Trunk

Control skin donors age range was 19–62 years.

3-acetate (TPA) as described earlier (Puri *et al.*, 1987). Forty-eight hours after seeding, the floating cells were aspirated and media containing  $10^{-11}$  M cholera toxin (CT) alone or supplemented with growth factors were added to the wells. Melanocyte counts were taken under phase contrast according to the method of Aubock *et al.* (1983). Skin from donors undergoing cosmetic surgery at our hospital were used for initiating normal human melanocytes in culture.

#### *Establishment of the PMR fetal lung fibroblast cell line*

A 5 month old male aborted fetus was obtained from the Department of Obstetrics and Gynaecology of our hospital. The lungs were removed under aseptic conditions and cut into small pieces. These were transferred to sterile petri dishes (Falcon, USA) and Eagles MEM was added. In about 2 weeks time when the fibroblast outgrowths could be seen under the microscope, the explants were removed and the cells allowed to reach confluency. They were maintained routinely in MEM.

#### *Extraction of growth factors*

The fibroblasts grown in plastic bottles (150 cm<sup>2</sup>, Corning) to confluency in MEM were collected by a rubber policeman and diluted to 1:1 with phosphate buffered saline (PBS). This suspension was sonicated twice for 15 s at 70 mA, diluted 10 times with PBS and centrifuged at 16,000 *g* for 20 min. This supernatant was then again centrifuged at 150,000 *g* for 45 min as outlined by Eisinger *et al.* (1985) for the isolation of growth factors from WI-38 cells. The proteins in this supernatant fraction were estimated by the method of Lowry *et al.* (1951). This extract diluted with PBS to various protein concentrations were added to melanocyte cultures.

#### *Chemicals*

Culture media used in the present study were purchased from GIBCO, Grand Island, USA. TPA was from Consolidated Midland Corporation, USA, while CT was from Sigma Chemicals Co., St. Louis, Missouri, USA. All other chemicals used were of the

*PMR-fibroblasts and PMR-GF*

As can be seen from figure 2, the newly established fetal lung cell line manifests all the characteristics of the fibroblast. The growth rates of these cells were comparable to other fetal lung fibroblast cell lines such as WI 38 investigated by others (Hayflick and Moorehead, 1961). PMR-GF diluted to final concentrations of 0.14  $\mu\text{g/ml}$  to 14.0  $\mu\text{g}$  protein/ml in the medium were tested on melanocytes obtained from perilesional skins of vitiligo subjects for their growth promoting activity. Dilutions of 2.8  $\mu\text{g}$  protein/ml of medium appeared to show optimum stimulus for growth and all further experiments were carried out at these concentrations. A non-specific inhibition of melanocyte growth rates were seen when the concentrations of PMR-GF were 14.0 and 5.6  $\mu\text{g}$  protein/ml in the medium (data not shown).

*Effect of PMR-GF on melanocytes of normal adult skins*

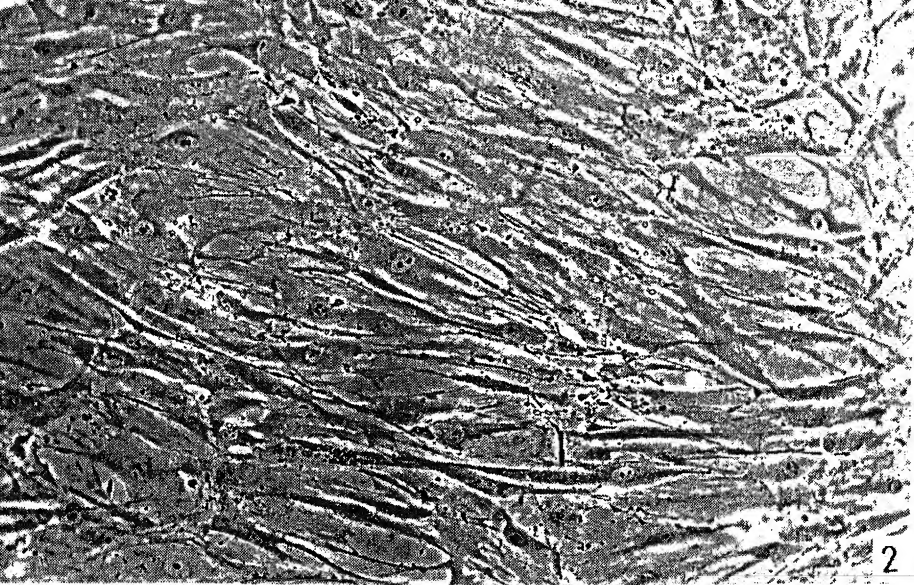
Melanocytes obtained from normal human truncal skin grew exponentially in the presence of  $10^{-11}$  M CT and 10 ng/ml TPA (figure 8). The cells grew individually evenly distributed throughout the culture dish (figure 4). Melanocytes cultured in the presence of PMR-GF manifested increased growth rates as compared to those grown in TPA and CT only. However, the increased growth rates could be seen 4–6 days after addition of PMR-GF (figure 8). Unlike in the case of melanocytes grown in presence of TPA and CT, those cultured in the presence of PMR-GF grew in clumps (figure 5).

*Effect of PMR-GF on perilesional skin melanocytes*

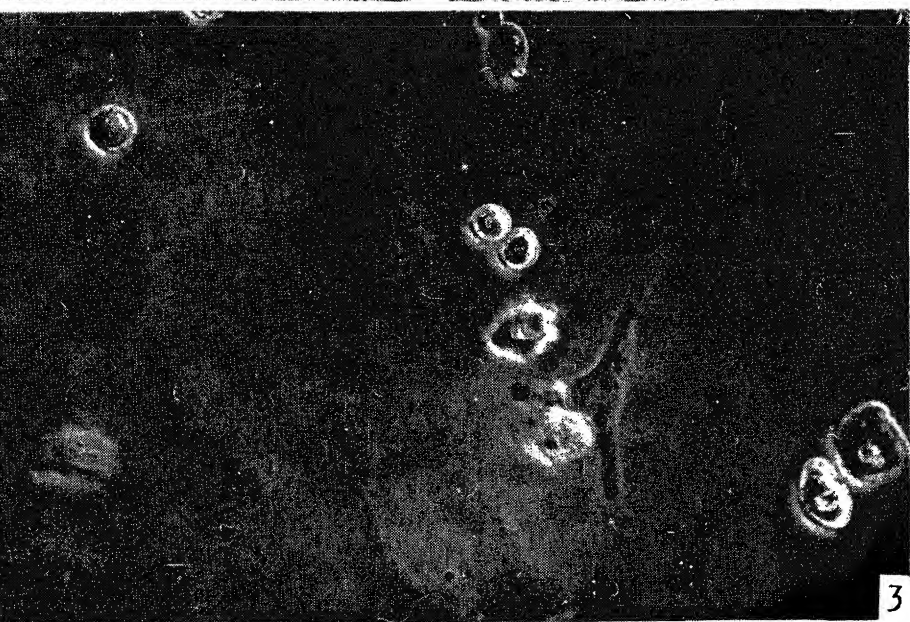
The melanocytes of the perilesional skins of vitiligo subjects failed to grow under standard conditions of  $10^{-11}$  M CT and 10 ng/ml TPA. They merely survived in culture for 15–30 days. Morphologically the melanocytes of the perilesional skin cultured in the absence of PMR-GF appeared to be larger in size and hypermelanotic as compared to the melanocytes of normal donors (figure 6). Exogenous addition of PMR-GF to these melanocytes induced in them a burst of growth 15 days after seeding epidermal cell suspensions. The cells soon reached a plateau phase and then started decreasing in numbers (figure 8). PMR-GF induced a reduction in size of the perilesional skin melanocytes and at the same time the cells appeared to be less hypermelanotic (figure 7). The clumping seen in normal human skin melanocytes in the presence of PMR-GF (figure 5) was also seen in the perilesional skin melanocytes (figure 7), but the extent of clumping was less.

*Effect of PMR-GF on the dendritic cells of vitiligo lesions*

The vitiligo lesions give rise to a few unpigmented dendritic cells, that survived for 8–10 days in culture (figure 3). The addition of PMR-GF at any concentration did

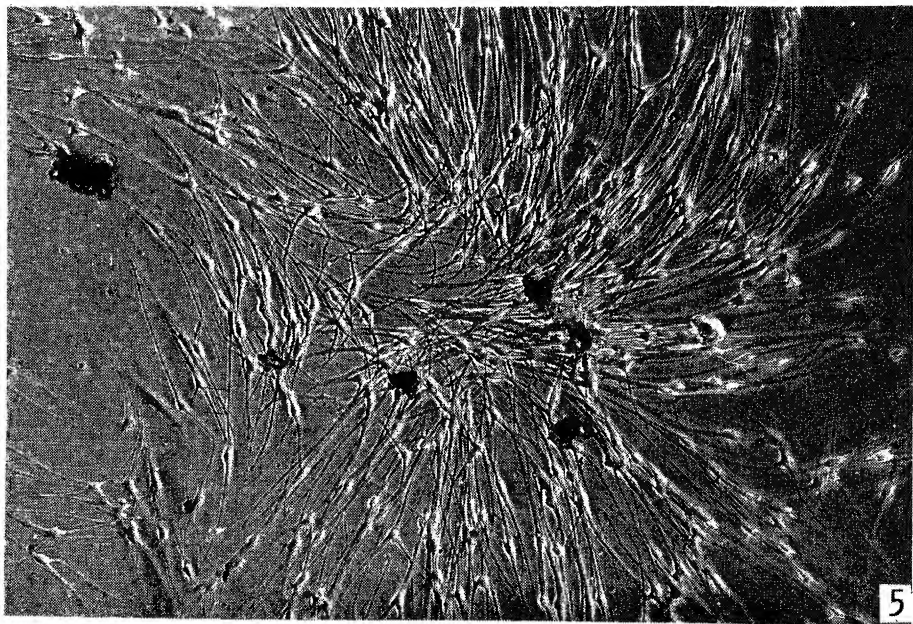
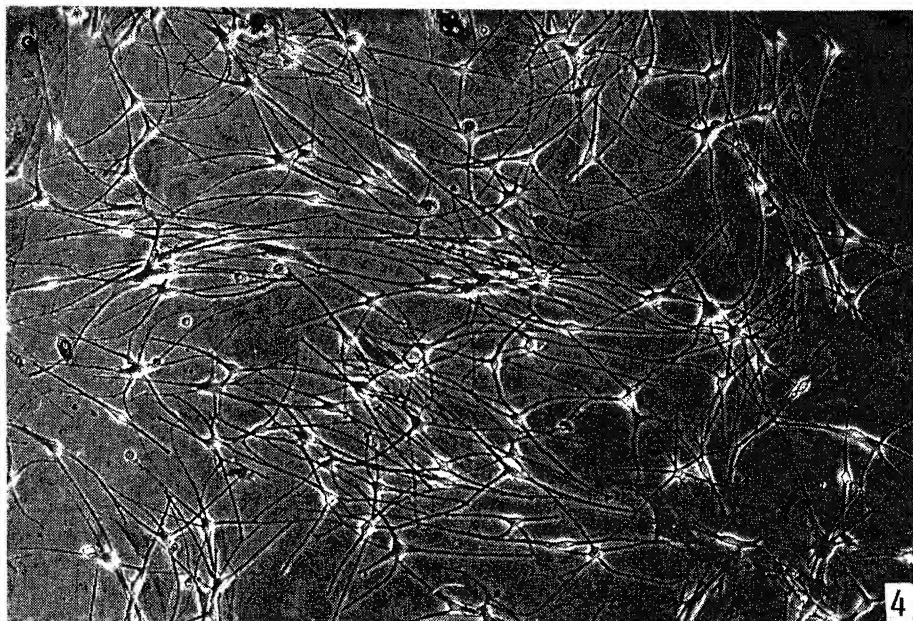


2



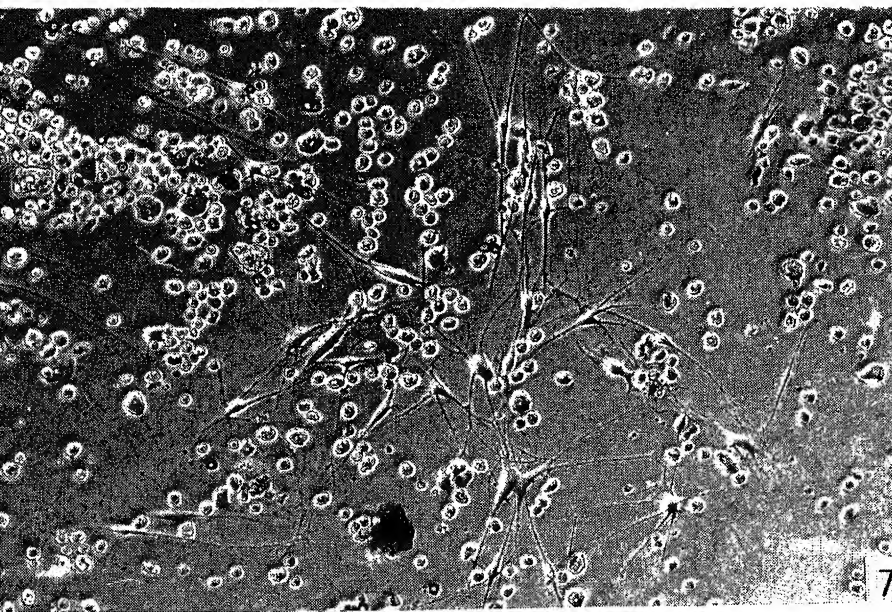
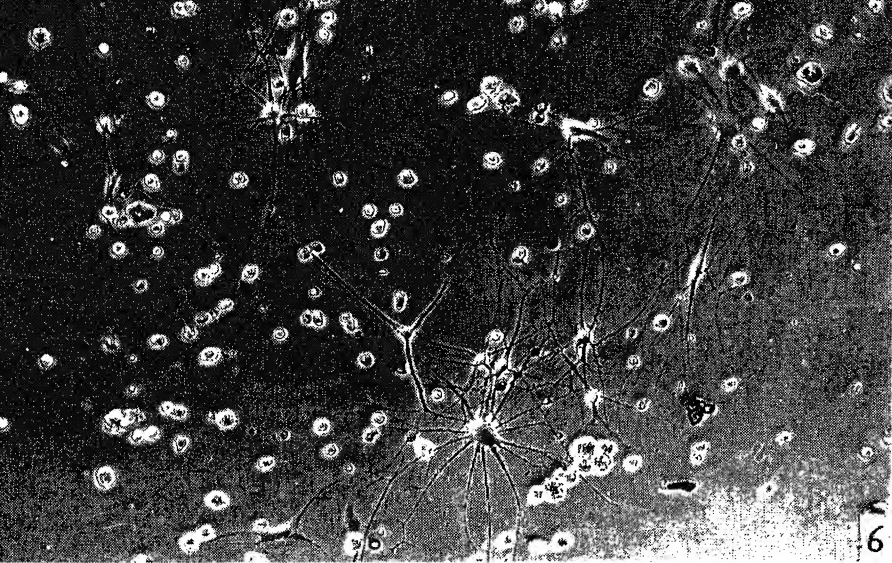
3

**Figures 2 and 3.** Cultured cells seen under phase contrast. 2. Fetal lung fibroblasts. 12th Passage X 92. 3. An unpigmented dendritic cell from a vitiligo lesion. Primary culture. X 370.



**Figures 4 and 5.** 4. Melanocytes from a normal donor growing individually. Primary culture X 92. 5. Melanocytes from the same normal donor cultured in the presence of PMR-GE. Primary culture X 92. Note clumping of the melanocytes.





**Figures 6 and 7.** 6. Melanocytes from the perilesional skin of a vitiligo subject. They are larger in size and also hyper-melanotic. Primary culture X 92. 7. The perilesional skin melanocytes of the same individual as shown in figure 6, but grown in the presence of PMR-GF. Primary culture X 92.



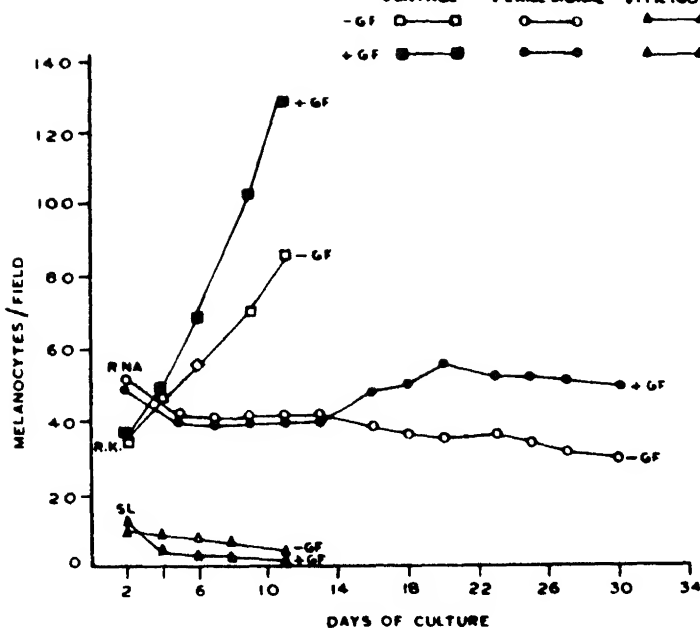


Figure 8. Growth kinetics of melanocytes and unpigmented dendritic cells of normal, perilesional and affected skins of control and vitiligo subjects grown in the presence and absence of optimum concentrations of PMR-GF.

## Discussion

Melanocytes constitute a small percentage of skin cells and have been found to undergo mitosis *in vivo* (Jimbow *et al.*, 1975). Only in 1982, it was possible to grow melanocytes *in vitro*. Tumor promoters such as TPA and other chemicals like CT were found to inhibit the attachment and proliferation of fibroblasts and keratinocytes and at the same time allow the growth of melanocytes (Marko and Eisinger, 1982). In addition, extracts of bovine hypothalamus (Gilchrest *et al.*, 1984), melanomas, astrocytomas and fetal lung fibroblast cell lines (Eisinger *et al.*, 1985) have been found to promote the growth of melanocytes in culture.

All the theories on the etiology of vitiligo suggest that melanocytes are absent in the affected lesion, being destroyed by various mechanisms (Ortonne *et al.*, 1983; Ramaiah, 1985). In most of these theories, the melanocytes themselves have been considered to be physiologically normal. We have recently shown, that melanocytes from adult healthy donors grow and can be trypsinized upto 3-4 passages in the presence of  $10^{-11}$  M CT and 10 ng/ml TPA while melanocytes cultured from uninvolved skins of vitiligo subjects, manifest a lag of 8-10 days for the onset of growth and cannot be passaged (Puri *et al.*, 1987). That the melanocytes present in skins of vitiligo subjects are not normal is also indicated by the present study. The melanocytes of the perilesional skins of vitiligo subjects fail to respond to growth factors extracted from fetal lung fibroblast cells in a manner that control donor skin mela-

Our results thus establish that the basic defect in human vitiligo lies with the melanocytes themselves. The anti-melanocyte antibodies seen in many vitiligo subjects could then be a secondary phenomenon (Naughton *et al.*, 1983). Recently, Boissy *et al.* (1986) have drawn similar conclusions based on the reduced proliferative capacities of melanocytes originating from neural crests of delayed amelanotic-myth (DAM-Smyth) chicken, an animal model of human vitiligo (Boissy *et al.*, 1983). Melanocytes originating from the neural crest cells of the DAM-Smyth chicken appear to become larger in size and hypermelanotic after a few passages in culture, a morphological phenomenon also seen in the perilesional skin melanocytes of human vitiligo subjects (figure 6). The PMR-GF induces a partial reduction in size and melanin content (figure 7) besides the small unsustained growth (figure 8). Whether other melanocyte mitogenic factors present in bovine hypothalamus (Gilchrest *et al.*, 1984), melanomas, astrocytomas and other fetal lung fibroblast cell lines (Eisinger *et al.*, 1985) would bring about complete normalization of these perilesional skin melanocytes remains to be investigated.

## Acknowledgements

This work was supported by a Research Grant from the Department of Science and Technology, New Delhi to A.R. M.M. is supported by Indian Council of Medical Research, New Delhi.

## References

- Boock, J., Kofler, D., Sifter, M. and Fritsch, P. (1983) *Br. J. Dermatol.*, **109**, 413.
- Boissy, R. E., Smyth, Jr. J. R. and Fite, K. V. (1983) *Am. J. Pathol.*, **111**, 197.
- Boissy, R. E., Moellmann, G., Trainer, A. T., Smyth, J. S. and Lerner, A. B. (1986) *J. Invest. Dermatol.*, **86**, 149.
- Eisinger, M., Marko, O., Ogata, S. I. and Old, L. J. (1985) *Science*, **229**, 984.
- Gilchrest, B. A., Vrabel, M. A., Flynn, E. and Szabo, G. (1984) *J. Invest. Dermatol.*, **83**, 370.
- Hayflick, L. and Moorhead, P. S. (1961) *Exp. Cell Res.*, **25**, 585.
- Kussain, I., Vijayan, E., Ramaiah, A., Pasricha, J. S. and Madan, N. C. (1982) *J. Invest. Dermatol.*, **78**, 243.
- Leibow, K., Roth, S. I., Fitzpatrick, T. B. and Szabo, G. (1975) *J. Cell. Biol.*, **66**, 663.
- Lerner, A. B. (1959) *J. Invest. Dermatol.*, **32**, 285.
- Lerner, A. B. and Nordlund, J. J. (1978) *J. Am. Med. Assoc.*, **239**, 1183.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Marko, O. and Eisinger, M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2018.
- Nishima, Y., Kawasaki, H. and Pinkus, H. (1972) *Arch. Dermatol. Forsch.*, **243**, 67.
- Naughton, G. K., Eisinger, M. and Bystryn, J. C. (1983) *J. Exp. Med.*, **158**, 246.
- Ortonne, J. P., Mosher, D. B. and Fitzpatrick, T. B. (1983) *Vitiligo and other hypomelanosis of hair and skin* (New York: Plenum Medical Book Co.).
- Prasad, N., Mojamdar, M. and Ramaiah, A. (1987) *J. Invest. Dermatol.*, **88**, (in press).
- Ramaiah, A. (1985) *Indian J. Dermatol. Venereol.*, **51**, 247.
- Schrober, P. (1941) *Dermatologica*, **84**, 257.



# Glycosaminoglycans in human fetal liver in relation to water and electrolytes

CHAMELI GANGULY and K. L. MUKHERJEE

Institute of Child Health, 11, Dr. Bireswari Guha Street, Calcutta 700 017, India

**Abstract.** The acidic mucopolysaccharides secreted into the extracellular space are thought to play many important functions amongst which are binding of water and electrolytes on the polyanionic glycosaminoglycans. Characteristically these components undergo continuous changes during growth and development of the fetuses. Relationships of the concentrations of glycosaminoglycans to the water and principal electrolytes at different periods of gestation were studied in human fetuses. It was found that during growth of the human fetuses there was a progressive decrease in water, thiocyanate space, total sodium content and glycosaminoglycans. However the decrease of glycosaminoglycans was greater than the rate of decrease of the other constituents. Hence mucopolysaccharides were thought to play more important roles than just binding of water and cations.

**Keywords.** Glycosaminoglycans; electrolytes; human fetuses; thiocyanate space.

## Introduction

A growing fetal organ like the liver and brain is characterized by growth and proliferation of endodermal or ectodermal cells in a matrix provided by mesenchymal tissue which supports and nourishes the characteristic cellular proliferation. The matrix consists of collagen fibres and proteoglycans secreted by the fibroblasts. It is envisaged that during growth of a fetal organ like the liver prior provision of the matrix ensures 3 dimensional proliferation of hepatic parenchymal cells. The matrix forms and fills the extracellular space, which is permeated by the collagen fibres and contains the nutrients, water and minerals in transit from the capillary to the tissue cells and the excretory products in the reverse direction. The nonfibrous part of the matrix consists mostly of proteoglycans which are proteins to which oligosaccharide chains consisting of repeating disaccharide units containing a hexosamine and an uronic acid (UA) are covalently attached. The structure and biosynthesis of proteoglycans have been extensively reviewed (Muir and Hardingham, 1983; Hascall and Hascall, 1981; Rodin, 1980). The oligosaccharide chains are known as glycosaminoglycans (GAGs). They are essential for maintenance of the structural integrity of many connective tissues and because of their characteristic polyanionic physico-chemical properties they have been implicated in a number of functions like binding of water, microions, distribution of various molecules by steric exclusion and cell-cell and cell-substrate interactions (Hook *et al.*, 1984; Comper and Laurent, 1978; Muir and Hardingham, 1975). During growth and differentiation of fetuses, there is a change in the water electrolytes and GAG contents of an organ like the brain (Cower, 1969; Margolis, 1969). The sodium content of the fetus was found to be

the adult exchangeable sodium was 40 mEq/kg in the fetus the corresponding value was 85 mEq/kg. This was attributed to a higher content of extracellular fluid in the fetus than in the adult. However, the relationship between water, electrolytes and GAGs has not yet been studied satisfactorily. The present paper was an attempt to study this relationship in human fetal liver at different gestation periods.

## Materials and methods

The fetuses were obtained from the Medical Termination of Pregnancy clinics of the Institute of Post Graduate Medical Education and Research, Calcutta. The program was cleared by the Ethical Subcommittee of the Institute, mothers gave written consent to the use of the fetuses for biological research. The authors had no say in the selection of cases for hysterotomy which was performed by the professor of obstetrics. Mothers who did not want to continue their pregnancies and wanted ligation at the same time formed the subject of the investigations. It was difficult to determine the exact period of gestation in some cases, because some mothers were vague about the last date of menstrual periods. Therefore, a combination of last date of menstrual period abdominal palpation and anthropometry of the fetuses was used to determine the period of gestation. All the fetuses were so called 'normal' fetuses and were delivered by hysterotomy. Sterile sodium thiocyanate solution (0.3 ml of 5% solution/kg) was injected to the mother 4 h before the conceptuses were taken out. Fetuses were put on ice in Dewar flask in the operation theater and brought to the laboratory where they were weighed and measured anthropometrically. They were dissected within 30 min after removal from the uterus. The entire liver was cut and used for the following estimations. Total water was measured by weighing and drying in an oven at 110°C for 18 h. Ashing of the dried specimen was carried out by incinerating in the muffle furnace at 550–650°C for 4 h. The weighed ashes were dissolved in 0.5 N HNO<sub>3</sub> and sodium and potassium concentrations were carried out by flamephotometry. Fetal blood was obtained by right atrial puncture into a heparinized syringe and immediately centrifuged to obtain the plasma. Aliquots of tissues were homogenised in the cold with water (1 ml/g) for determination of the thiocyanate space according to Eder (1951). Extraction of GAGs and their fractionation were carried out by the method of Singh and Bachhawat (1968). UA was determined in the dialyate by the method of Dische (1947) as modified by Bitter and Muir (1962). UA was multiplied by 2.4 to get an approximate value of GAG content. GAGs were fractionated according to the method of Schiller *et al.* (1961). Hexosamines in the fractions of GAGs were hydrolyzed in 6 N HCl in sealed ampoules and the hydrolysate were dried over NaOH pellets in the vacuum desiccator. The dried samples were dissolved in a small volume of water in which the hexosamines were determined by the method of Ludowicz and Benmaman (1968). Total sulphate content was measured by the method of Dodgson and Price (1962). Glucuronolactone, acetylacetone, gelatine, fructose-6-phosphate, glutamine, glucosamine, galactosamine, hyaluronic acid (HY), chondroitin-4-sulphate, chondroitin-6-sulphate, sodium salt of heparin were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. All other chemicals were of analytical grade and bought from British Drug House or E. Merck. Solvents were redistilled.

for the sake of convenience the fetuses have been divided into 6 groups—A, B, C, D, and F (table 1) according to a difference in the gestation period of 4 weeks. The gestational age was ascertained from the date of the last menstrual period, the body weight of the fetuses, the crown rump and crown heel lengths. Fetuses of group A included gestational ages of 9–12 weeks; their body weights were from 1.1–14.5 g. Group B represented fetuses of 13–16 weeks with body weights of 14.6–110 g. Group C included fetuses of 17–20 weeks gestation with body weights of 111–315 g. Group D included fetuses of 21–24 weeks with body weights from 360–680 g. Group E contained fetuses of 25–28 weeks with body weights from 681–1050 g. Another group represented specimens of body weight 900–3000 g including premature and full term babies from 1–8 days of age. Of the 115 fetuses investigated in the present series 15 belonged to group A, 26 to B, 36 to C, 21 to D, 14 to E and 3 to F.

**Table 1.** Grouping of fetuses and weight length (crown-rump) relationship.

Group	No. of fetuses	Week	Weight (g)	Length (mm)	Length change
					Weight change
A	15	9–12	1.5–14.5	25–58	4.6
B	26	13–16	15.6–108	62–109	1.1
C	36	17–20	115–295	110–157	0.90
D	21	21–24	330–660	163–211	0.37
E	14	25–28	715–1025	203–252	0.20
F	3	29–32	1055–1650	230–277	0.17

### *Weight of the liver*

Table 2 and figure 1 shows the weights of the fetal livers at different periods of gestation, expressed as the total weight of the organ and in figure 2 as g per kg of body weight. With progress of gestation, there is an increase in the weight of the

**Table 2.** Weight of liver.

Weight of fetus (g)	No. of samples	(g/kg body wt.)	
		Mean $\pm$ S.D.	Mean $\pm$ S.E.M.
0–100	37	47 $\pm$ 8.8	47 $\pm$ 1.5
100–200	19	40 $\pm$ 3.6	40 $\pm$ 0.83
200–300	14	39 $\pm$ 2.5	39 $\pm$ 0.74
300–400	9	38 $\pm$ 3.1	38 $\pm$ 1.0
400–500	4	40 $\pm$ 2.9	40 $\pm$ 1.5
500–600	4	35 $\pm$ 2.2	35 $\pm$ 1.1
600–700	3	42 $\pm$ 7.2	42 $\pm$ 4.2
700–800	4	41 $\pm$ 5.3	41 $\pm$ 2.7
800–900	2	39	39
900–1000	3	33 $\pm$ 3	33 $\pm$ 1.1
1000–2000	6	39 $\pm$ 4.2	39 $\pm$ 1.1

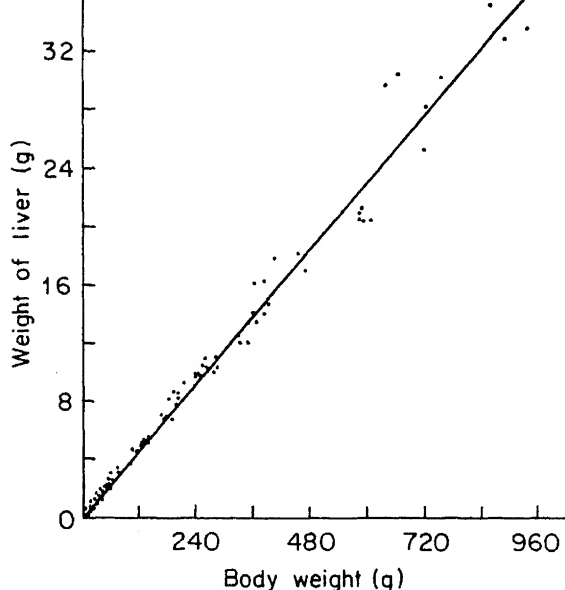


Figure 1. Total weight of human fetal liver.

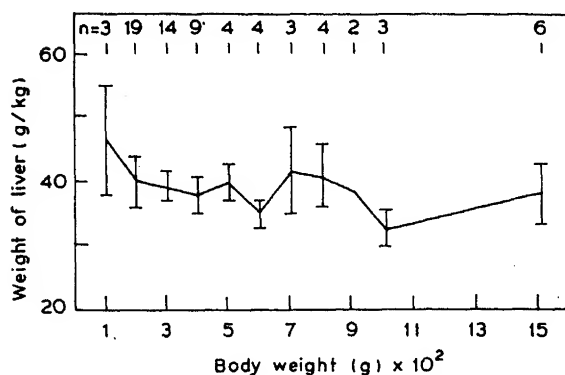


Figure 2. Weight of human fetal liver (g/kg of body weight).

liver. But the rate of increase is not the same at the different ages. When the weight of the liver is expressed as g per kg of body weight, it is seen that except at the earliest period investigated, *i.e.* at 9–12 weeks of gestation the weight of liver was almost exactly proportional to the weight of the fetus at all other ages, even upto 8 days after birth. It forms about 4% of the total body weight. At 9–12 weeks of gestation the liver was comparatively a bigger organ. In the fetuses of very early gestation period, the liver constituted 5.5% of body weight. Subsequently from about 12 weeks

dehydrated defatted tissue was digested with papain followed by alkali and the proteins were precipitated with trichloroacetic acid (TCA). UA was determined in the dialyzed supernatants. Results are shown in table 3.

**Table 3.** Total MPS of human fetal liver of different gestation period.

Group	Age (weeks)	No. of fetuses	Total UA mg/g dry defatted tissue
B	13-16	3	0.74 ± 0.16
C	17-20	10	0.65 ± 0.07
D	21-24	10	0.56 ± 0.07
E	25-28	5	0.55 ± 0.09
Postmortem	0-8 days	6	0.35 ± 0.02

The amount of liver obtainable in fetuses of 9-12 weeks gestation was not enough to process extracts for total MPS and subsequent fractionation. The total UA content per g of dried defatted liver in the fetuses of gestation period from 13-32 weeks was not different and the difference between the groups was not statistically significant. Although the MPS content of the fetal liver did not change much during the progress of gestation, its amount became reduced within the first few days of life. Whereas the hexuronic acid content of fetal liver varied from 0.5-0.7 mg/g, in post-natal life the amount was around 0.3 mg/g.

#### *Fractionation of MPS in developing human liver*

The MPS in 0.04 M NaCl was precipitated by cetyl pyridinium bromide (CPB) (3 mg per mg of MPS) and centrifuged after the addition of celite (20 mg/mg of MPS). The precipitation was judged complete when hexuronic acid was found to be absent in the supernatant. The precipitate was washed with small volumes of 0.04 M NaCl containing 0.1% CPB. The washings were checked for any hexuronic acid contents, which were found to be absent. Thus the precipitation of MPS in the remaining extract was treated as being quantitative.

The CPB polysaccharide celite complex was extracted repeatedly with small volumes of 0.4 M NaCl containing 0.1% CPB until the supernatant after centrifugation was free of hexuronic acid. This fraction (fraction 1) usually contains hyaluronate. However, in this eluate, variable amounts of chondroitin sulphates and possibly heparan sulphate too may be eluted. The eluates were, therefore, analysed for glucosamine, galactosamine and sulphate. In hyaluronate the ratio of glucosamine to UA should be 1, there should be no galactosamine or sulphate, both of which are constituents of chondroitin sulphate (CS). Results are shown in table 4.

In group A, the livers were too small to be analyzed. In group B, we could determine the total amount of UA in fraction 1, i.e. the MPS extractable in 0.4 N NaCl, but the determination of the different hexosamines and sulphates could not be undertaken in the small amount of material available. In group B and C the total amount of fraction 1 was found to be same; the hexosamine present was mostly



**Table 4.** Composition of MPS of human fetal liver (Fraction-1).

Group	Age (weeks)	No. of specimens	UA mg/g dry defatted tissue	Hexosamine*	Glucosamine*	Galactosamine*	Sulphate*
B	13-16	3	0.40				
C	17-20	12	0.40 ± 0.02	1.30	1.27	0.03	0.9
D	21-24	10	0.30 ± 0.05	1.25	1.02	0.23	1.0
E	25-28	5	0.28 ± 0.08	0.90	0.75	0.15	0.8
F	29-32	—	ND	ND	ND	ND	ND
Postmortem	0-8 days	7	0.15 ± 0.02	0.75	0.60	0.15	1.37

\*Expressed as molar ratio of UA.

ND, Not done.

glucosamine but the content of sulphate was almost equal to that of the UA. Thus this fraction contained the nonsulphated HY, some amount of heparitin sulphate and small amount of CS. In groups B and E, total MPS slightly decreased and this fraction also contained predominantly HY but considerable amounts of chondroitin and heparan sulphate was found to be eluted in this fraction. In post mortem specimens of babies of 0-8 days ages, the total amount of fraction 1 was found to be much less than in the fetal specimens but the nature of the material eluted in this fraction was observed to be a mixture of HY and CS and heparitin sulphate.

The CPB polysaccharide complex which remained after fraction 1 was eluted out of the complex was next extracted with small volumes of 1.2 M NaCl containing 0.1% CPB, until no more hexuronic acid was eluted. This fraction should contain CS. In order to check whether this fraction contained other MPS too, this fraction was checked for glucosamine, galactosamine, and sulphate. In CS, the aminosugar is Glam and the ratio of UA to sulphates is less than one depending on the sulphonation of the hexosamines. Results are shown in table 5. In group B, the fraction was insufficient in amount for determination of various constituents. In group C, the amount of fraction II as determined by the total hexuronic acid content was higher than in the livers of babies dying after 0-8 days of life. When the fraction was, however analyzed for the glucosamine and galactosamine contents, most of the aminosugar was found to be galactosamine rather than glucosamine, which should be the preponderant aminosugar in this fraction.

The CPB-polysaccharide complex was next eluted with 2.1 M NaCl containing 0.1% CPB in order to elute the heparin. Results are shown in table 6. The content of heparin was low as compared to fraction 1 and 2 but by and large the heparin

**Table 5.** Composition of MPS of fetal liver (Fraction-2).

Group	Age (weeks)	No. of cases	UA mg/g dry defatted tissue	Hexosamine*	Glucosamine*	Galactosamine*	Sulphate*
B	13-16	3	0.26				
C	17-20	12	0.21 ± 0.02	0.97	0.13	0.84	0.87
D	21-24	10	0.24 ± 0.04	1.20	0.13	1.07	1.5
E	25-28	5	0.19 ± 0.07	0.86	0.38	0.48	0.98
Postmortem	0-8 days	7	0.16 ± 0.02	0.70	0.20	0.50	0.98

**Table 6.** MPS in human fetal liver (Fraction 3).

Group	Age gestation	No. of fetuses	UA mg/g dry defatted tissue	Hexosamine*	Glucosamine*	Galac-tosamine*	Sulphate*
	12-16	3	0.08 ± 0.04	ND	ND	ND	ND
	16-20	9	0.04 ± 0.02	0.88	0.44	0.44	1.0
	20-24	8	0.02 ± 0.02	0.90	0.77	0.15	3.0
	24-28	7	0.07 ± 0.02	0.71	0.68	0.03	3.1
(Postmortem)	0-8 days	6	0.040 ± 0.02	1.2	1.1	0.1	1.0

Expressed as molar ratio of UA.

ND, Not done.

content in fetal life was slightly greater in Group B and E than in postmortem liver in postnatal life. This fraction also cannot be considered to consist of heparin only as considerable amount of galactosamine was present. The sulphate content was characteristic of heparin in groups D and E but low in group C and postmortem.

#### *Water and electrolyte content of human fetal liver*

Water content of human fetal livers at different periods of gestation is shown in Table 10. It varied from 80.5% of the organ weight at 9-12 weeks to 77.6% at 25-28 weeks. The decrease was progressive except in the last two groups where the water content was almost unchanged. We could obtain livers of 4 children who died from prematurity and other causes; the water content in them was also like that at 21-28 weeks, namely around 78%. The water content of adult livers obtained by surgical autopsy was around 69% of organ weight. Measurement of extracellular water has been attempted with many compounds like inulin, sucrose, sodium thiocyanate (NaSCN), radioactive  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{So}_4^-$  etc and each of these substances have their own uses and defects (Deane *et al.*, 1951; Gaudino and Levitt, 1949). Originally, we tried to use inulin to determine extracellular space (Deane, 1951) but we ran into troubles. The determination of inulin concentration in the blank became a serious problem and we had to abandon the method in favour of a substance like NaSCN which in fetal tissues had a zero blank value. Briefly, the method consisted of injecting sterile solutions of sodium thiocyanate into mother and carrying out hysterotomy 4 h later. Table 7 shows thiocyanate concentrations of different fetal fluids after maternal injection of 0.3 ml NaSCN (5% in saline)/kg of body weight. The concentration of thiocyanate in maternal serum was higher than that of fetal serum. However, fetal serum concentration was more uniform than the maternal. The amniotic fluid, fetal bladder fluid

**Table 7.** Thiocyanate concentrations in body fluids.

		mg%
Maternal serum	(9)	4.7 ± 0.91
Fetal serum	(9)	2.7 ± 0.21
Amniotic fluid	(3)	0.48, 0.22, 0.94
Fetal bladder fluid	(2)	0.81, 0.48

and fetal cerebrospinal fluid had lower concentrations than fetal serum. Since thiocyanate was found to be permeable across the placenta and since we assumed that fetal circulation was greater and fetal urinary excretion was slower than the adult, we presumed that thiocyanate had reached equilibrium concentration in 4 h. For determination of thiocyanate in the liver, the organ was homogenized in water and proteins were precipitated with same volume of 10% TCA. Thereafter the method was followed as described for the plasma by Eder (1951). The extracellular space per g of tissue was calculated according to the formula extracellular fluid =  $x/y \times 100 \times 1.1$  where  $x$  and  $y$  were thiocyanate concentrations in mg per cent in the organ and plasma respectively and 1.1 was a constant for Donnan Equilibrium. Total water was calculated by drying the tissue to constant weight at 110°C. The intracellular water taken as the difference of total organ water and extracellular space. Results are shown in table 8.

**Table 8.** Water content and distribution of human fetal liver.

Body weight	Approx. gest. period	Total water (g%)	Extracellular (g%)	Intracellular (g%)
95	16	80	53	27
101	16	79	51	28
107	16	79	51	28
120	17	80	53	27
250	20	81	45	36
330	23	79	46	33
375	24	80	46	34
402	25	79	46	33
877	27	78	43	35

The thiocyanate space varied from 53 ml per 100 g of liver at 16 weeks of gestation to 43 ml at 27 weeks. As the total water did not change very much during the different gestation period studied the corresponding intracellular water (*i.e.* total water-thiocyanate space) increased from 27 ml per 100 g at 16 weeks to 35 ml at 27 weeks. Whether the difference observed was due to any peculiarity of thiocyanate movement in fetal liver or to real movement of water it is difficult to say. Such an increase in intracellular water at later periods of gestation cannot be ascribed to a greater number of cells per unit area at this time, because the DNA content per 100 g of liver tissue was higher at earlier period of gestation than at later periods. The increase in intracellular water might be due to an increase of volume and mass of the individual cells, since the total protein concentration per g of tissue was found to be higher at later periods of gestation than at earlier periods.

#### *Thiocyanate space in human adult liver*

Adult women undergoing operation for cholelithiasis were the subjects of the experiments. Informed consent was taken from these patients about the nature of experiments. Sterile sodium thiocyanate (15 ml of 5% solution) was slowly injected intravenously 4 h before the operation. At the time gall bladder was being taken out a sample of

ions in the serum and the specimen of liver were measured. The total amount of water was also determined in an aliquot of the liver sample. Results are shown in table 9. In the determination of thiocyanate concentration in the TCA extract of liver, a blank determination was found to be essential which contained the equivalent amount of TCA filtrate and 1.5 ml of 2.5%  $\text{HNO}_3$ . A further blank was also necessary to correct for nonspecific substances giving colour with ferric nitrate (8% of the SCN space). The thiocyanate space varied from 30–35% and intracellular water from 34–42% of the wet weight of the liver.

**Table 9.** Thiocyanate space in female human adult liver.

Specimen No.	Age (years)	Total liver water	Thiocyanate concentration in serum (mg%)	Thiocyanate space (ml/100 g)	Intracellular water (ml/100 g)
1	46	72.5	4.6	30.0	42.5
2	39	69.3	5.1	35.0	34.3
3	48	70.0	5.2	35.0	35.0

In the 3 specimens, the thiocyanate space varied from 30–35% and the intracellular water (total water–thiocyanate space) 34.3–42.5%. None of the livers showed any histological abnormality.

### *Sodium and potassium contents of the human fetal liver*

The metabolism of water, electrolytes and MPS is closely interrelated. The matrix containing the polyanionic acidic mucopolysaccharides (AMPs) is supposed to attract the hydronium ions and the common cations like sodium and potassium ions. The more charged it will be, the more will it attract the cations which again, in their turn, will bring the anions like the chloride and bicarbonate around them. We, therefore, determined the sodium and potassium concentrations in the ashed material of the liver. Results are shown in table 10. Potassium concentration in mEq/kg body weight varied from 41–51; virtually there was no change at different periods of gestation. Sodium content, of the liver, however, in earlier periods of gestation *e.g.* group A and B was higher than in later periods.

**Table 10.** Total water, ash, sodium and potassium contents of human fetal liver.

Group	Gest. period (weeks)	Water (g%)	Ash (g%)	K <sup>+</sup> (mEq/kg)	Na <sup>+</sup> (mEq/kg)
A	9–12 (7)	80.7 ± 1.2	1.62 ± 0.93	41 ± 5	94 ± 10
B	13–16 (10)	79.4 ± 0.5	1.90 ± 0.13	43 ± 4	71 ± 7
C	17–20 (20)	78.5 ± 0.3	1.63 ± 0.02	51 ± 2	67 ± 8
D	21–24 (11)	78.3 ± 0.6	1.68 ± 0.15	48 ± 5	60 ± 10
E	25–28 (8)	77.6 ± 0.4	1.31 ± 0.18	44 ± 4	66 ± 10
Postmortem	0–8 days (4)	78.2 ± 1.3	1.57 ± 0.19	44 ± 3	62 ± 3
	8 (Adult)	69.0 ± 1.6	1.52 ± 0.07	57.3	63 ± 12

and potassium concentrations were determined by subtracting the extracellular ions from the total ions. Results are shown in table 11. The thiocyanate space decreased from 520 ml/kg of the organ weight at 17 weeks to 460 ml/kg at 28 weeks. The intracellular water kept more or less constant at 32%. The total sodium of the fetal liver was all in the thiocyanate space. Actually the calculated extracellular sodium was found to be higher than the total sodium content, so that it may be assumed that there was little sodium in the intracellular space. In contrast, in the adult liver about 13% of sodium content could be calculated to be within the extracellular compartment. Potassium was chiefly intracellular. In most instance the osmolar equivalent of intracellular and extracellular fluid calculated from the sodium and potassium concentrations matched exactly.

## Discussion

### *Growth of liver at different gestational periods*

In a growing organ like the human fetal liver, the term growth envisages both increase in cell number and increase in cell mass. The two processes are probably not synchronous. In the early stages of gestation the organ may be preoccupied with increase in cell number and in the later stages with both numerical as well as dimensional increase.

Both these two types of growth involves utilization of nutrients. In postnatal life the nutrients come from outside and are subject to external control. In utero, however, the different nutrients are derived from the mothers' circulation and in normal pregnancies in healthy mothers they are presumably not in deficient supply. Consequently the growth of the liver in utero can be envisaged as an intrinsic process, dictated by the genotype. In the early stages *e.g.* between 9–12 weeks of gestation the liver forms a greater percentage of body weight than at later stages. This may be due to two reasons. At this time, the other organs of the body, at least the bulkier ones like the muscles, bones and brain are comparatively smaller so that relatively a greater percentage of body weight is constituted by the liver. Or the rate of division of individual liver cells may be very high in this stage so that the organ is relatively a big one. It appears that both of these statements are true. At very early stages of development, *e.g.* at 7 weeks, the abdominal wall is open; when it begins to close, the space is not enough for retaining all the abdominal organs inside; thus the elongating intestinal loop herniates into the umbilical cord and returns only when the abdominal cavity has increased in size both absolutely and relatively. The relative increase of the abdominal cavity is due to a decrease in the rate of cell division of the liver. But, later on, from 12 weeks onwards the weight of the liver bears an almost constant proportion to the body weight. It appears that the liver subserves vital metabolic functions for the controlled growth of the whole fetus. Hence as the fetus grows, the liver also grows in proportion to the whole fetus. But this is only a teleological explanation; we are totally ignorant of the forces which control the growth of any organ, let alone the liver.

**Table 11.** Sodium and potassium concentrations in human fetal liver (in different compartments) values are expressed in wet tissue.

Gr.	Gest. period (weeks)	Water space (%)	CNS space (ml/kg)	Intra- cellular water (ml/kg)	Plasma		Extra- cellular liver		Intra- cellular liver		Intra- cellular liver	
					Na <sup>+</sup> (mEq/L)	K <sup>+</sup> (mEq/L)	Na <sup>+</sup> (mEq/kg)	K <sup>+</sup> (mEq/kg)	Na <sup>+</sup> (mEq/kg)	K <sup>+</sup> (mEq/kg)	Na <sup>+</sup> (mEq/kg)	K <sup>+</sup> (mEq/kg)
C	17-20 (4)	79.5	520	275	140	11.6	79.1	6.6	0.0	44.4		
D	21-24 (4)	78.5	465	320	139	10.1	70.6	5.2	0.4	42.8		
E	25-28 (4)	78.5	460	325	135	12.5	68.4	6.3	0.6	37.7		
Adults	(8)	69.0	320	370	142	4.8	49.9	2.0	7.1	55.0		

Intracellular water = Total water - CNS space.

Extracellular Na = Plasma Na  $\times$  1.1 (Donnan Eq)  $\times$  CNS space.

Extracellular K = Plasma K  $\times$  1.1 (Donnan Eq)  $\times$  CNS space.

Intracellular Na = Total sodium - extracellular sodium.

Intracellular K = Total potassium - extracellular potassium.

In almost all fetal organs, the epithelial elements in their developmental process becomes surrounded by mesenchymal tissue. The mesenchyme is an extraordinarily versatile tissue with many potentialities which find expression under the varied conditions offered during the course of development. Thus they may give rise to connective tissue, cartilage, bone, blood, smooth muscle and endothelium. Of these various derivatives, the connective tissue performs a predominantly mechanical function of support. This supporting function is exhibited by a composite of 3 things, a ground substance, some protein fibres and fibroblasts. The former two are products of the fibroblasts. The ground substance is considered to be structureless (by light microscopy) and nonliving and to be the actual component, responsible for the characteristic mechanical function. The maintenance of the ground substance is a function of the associated specialized cells in active condition.

In any developing organ, including the liver, the mesenchymal cells at first form a compact layer around the epithelial out growth. However, some mesenchymal cells are found to be loosely arranged around the mesenchymo-epithelial tubes forming a sort of labyrinthine space. They secrete the ground substance consisting of MPS's and collagenous fibres. The MPS are thought to be structureless; but the structurelessness refers to light microscopy only; molecule-wise the MPS's form a group of highly oriented organized molecules.

Just as the mesenchymal cells can take up various lines of differentiation, like the connective tissue, cartilage, bone, blood or endothelium, the characteristic cell of the connective tissue, *i.e.* the fibroblast can secrete many different kinds of molecules into the interstitial space. One such class of molecules is the MPS, the ones that we are dealing with here are called AMPs; they contain hexuronic acid. The amount of hexuronic acid in the extracted, partially purified MPS material is, therefore, a measure of the total MPS of an organ. In embryos the mesenchyme is principally cellular but in fetuses the matrix is quantitatively greater than the actual cellular element. The higher UA in fetal organs of early gestation period may be due to a relatively greater content of mesenchymal cells in a given volume of tissue. It must, however, be cautioned that these cells are relatively undifferentiated and less ready to extrude the proteoglycans in the interstitial space.

The procedure adopted for fractionation of the MPS of human livers was not ideal because none of these fractions were pure classes of compounds. Fraction 1, comprising HY should not contain Galm and sulphate. Although Galm content was proportionally low, there was quite a large amount of sulphate. It appeared, therefore, that some amount of CS and heparan sulphate was present in this fraction. There was a diminution in the amount of fraction 1 per g of dry defatted tissues, at later periods of gestation and in immediate postnatal life. Fraction 2 was also not a pure class of compounds inasmuch as in this fraction a large quantity of glucosamine was found. The sulphate content more or less corresponded with an equimolar quantity of hexosamine. The contamination might therefore, be either due to heparitin sulphate or even to heparin. The quantity was almost half of that found in fraction 1. Fraction 3 constituted less than 10% of the total UA content and the fraction also contained a mixture of a variety of compounds. If fraction 3 constituted entirely of heparin, it should have a hexuronic acid ratio to sulphate more than 2. However in the earlier

part. The low sulphate may be due to a defect in the isolation procedure whereby mucopolysaccharides of low sulphate content like that of CS might have crept in. During the progress of gestation there was a decrease in the total UA content of the liver; the decrease was mostly in the HY fraction. We do not know the contribution of the individual proteoglycans to the overall function of the MPS. The decrease in the HY fraction with presumably a greater stability of the matrix specially in extra-uterine life would go against the indispensability of HY to form a stable structure with link protein for the other polysaccharides.

#### *Water and electrolytes of the human fetal liver*

The main difference in the sodium and potassium concentrations of the serum in fetal and adult sera consisted in the higher serum potassium content in fetal life. Whereas adult serum potassium concentrations were  $4.8 \text{ mEq/L} \pm 0.5$ , the corresponding concentrations in fetal sera were  $10.8 \text{ mEq/L} \pm 1.3$ . Since there was no difference in the sodium contents of fetal and adult sera, the total cation concentration and the total osmolarity of fetal sera were higher than those of adult sera. This higher osmolarity of fetal water probably has something to do with the floatation characteristic of the fetus in the amniotic fluid. The density of a few smaller fetuses was found to be around 1.103 by water displacement method, whereas that of the amniotic fluid was around 1.117. The maintenance of this density was a function of the osmotic tension of the body fluids of the fetus. A teleological explanation of the higher serum potassium in fetal life may also be given. A growing organ actively synthesizing protein and depositing the protein in the tissues needs potassium. The high serum potassium level in fetal life ensures a liberal supply. Furthermore, potassium concentration in the intracellular fluid of fetal liver was lower than that of the adult liver. Perhaps, the sodium pump in the tissue is not as effective in fetal liver as in adult liver so that potassium leaks out into the extracellular fluid.

The thiocyanate space in fetal liver was greater than in adult liver. The extracellular fluid was therefore present in greater quantity in fetal life than in adult life. In fact, the higher total water content of fetal liver may be ascribed entirely to the greater quantity of extracellular fluid. The intracellular fluid was not increased in fetuses. This huge amount of extracellular fluid is not present as free water but must be in a soluble form in combination with a macromolecule. The macromolecule is not presumably a protein but a polysaccharide, a MPS to be exact. Total MPS contents (table 3) was higher in earlier gestation period than in later. The decrease was still more manifest in neonatal livers on postmortem examination. The total water content of fetal liver (table 8) was lower than that of the adult liver. However, the decrease in total MPS from fetal to neonatal liver was more than the decrease in total water from fetal to adult life. It might therefore, be assumed that among the various functions of the MPS, only one function is related to its binding property with water. There are other functions of these classes of compounds, which are perhaps, more important in fetal life. The decrease of MPS during progress of gestation was almost entirely ascribable to a decrease in hyaluronates. Since the binding of hyaluronate to water is a well known phenomenon, the relation between the decrease of hyaluronate content and water is more than coincidental.



The presence of a high amount of water in the extracellular space necessitates retention of salts to maintain osmotic equilibrium. The total cation content of the extracellular fluid per kg of organ weight in fetal liver amounted to 75 mEq whereas in the adult liver it was only 52 mEq. The increase of extracellular water in fetal liver as compared to adult liver was between 50 and 70%. Therefore, the total cation content in extracellular fluid of fetal liver was increased relatively as well as absolutely. The total MPS contents of fetal liver in earlier gestation periods was about double that of neonatal livers. Hence the MPS must play important role in the greater binding of not only water but of cations as well.

Moreover, the other important functions of GAGs like interchain binding (Fransson, 1976), binding to collagen (Toole, 1976), platelet factor 4 (Moore *et al.*, 1975) and in cell-cell and cell-substrate interactions (Hook *et al.*, 1984) may determine the behaviour of the matrix polysaccharides.

## Acknowledgements

Grateful acknowledgements are due to Dr. B. K. Bachhawat, Department of Biochemistry, Delhi University, Delhi for assistance and encouragement and to Dr. Narayan Chaudhury, Professor of Obstetrics, Institute of Postgraduate Medical Education and Research, Calcutta for providing fetuses of patients. The authors are also grateful to the Indian Council of Medical Research, New Delhi for financial assistance.

## References

- Behrman, R. E. and Vaughan, V. C. (1983) in *Nelson's Textbook of Pediatrics*, 12th edition (Philadelphia: W. B. Saunders) p. 210.
- Bitter, T. and Muir, H. (1962) *Anal. Biochem.*, **4**, 330.
- Comper, W. D. and Laurent, T. C. (1978) *Physiol. Rev.*, **58**, 255.
- Deane, N. (1951) *Methods Med. Res.*, **4**, 48.
- Deane, N., Schreiner, G. E. and Robertson, J. S. (1951) *J. Clin. Invest.*, **30**, 1463.
- Dische, Z. (1947) *J. Biol. Chem.*, **167**, 189.
- Dodgson, K. S. and Price, R. G. (1962) *Biochem. J.*, **84**, 106.
- Eder, A. (1951) *Methods Med. Res.*, **4**, 48.
- Fransson, L. A. (1976) *Biochem. Biophys. Acta*, **437**, 359.
- Gaudino, G. and Levitt, M. F. (1949) *Am. J. Physiol.*, **157**, 387.
- Hascall, V. C., Hascall, G. K. (1981) in *Cell Biology of Extracellular Matrix*, (ed. E. D. Hay) (New York: Plenum) p. 39.
- Hook, M., Kjellen, L. and Johansson, S. (1964) *Annu. Rev. Biochem.*, **53**, 847.
- Ludowicz, J. and Benmaman, D. (1968) *Carbohydr. Res.*, **8**, 185.
- Margolis, R. U. (1969) *Handb. Neurochem.*, **1**, 245.
- Moore, S., Pepper, D. S. and Cash, J. D. (1975) *Biochim. Biophys. Acta*, **379**, 370.
- Muir, H. and Hardingham, T. E. (1975) *MTP Int. Rev. Sci. Biochem. Ser. 1*, **5**, 1.
- Muir, H. and Hardingham, T. E. (1983) in *Biochemistry of carbohydrates*, (ed. W. H. Whelan) (London: Butterworths) p. 153.
- Rodin, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans*, (ed. W. J. Lennerz) (New York: Plenum) p. 267.
- Schiller, S., Glover, A. and Dorfman, A. (1961) *J. Biol. Chem.*, **236**, 983.
- Singh, M. and Bachhawat, B. K. (1968) *J. Neurochem.*, **15**, 249.

# Antigenic components of human glioblastoma multiforme and rat C<sub>6</sub> glioma using monoclonal antibodies

ELA SHARMA, KULVINDER S. KOCHHAR and  
SHAIL K. SHARMA\*

Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110 029, India

**Abstract.** With whole U87MG cells used as antigenic stimulant, two clones 1A5G6 and 1D3A3 secreted monoclonal antibodies which gave intense staining in monolayer cultures of the cells as ascertained by indirect immunofluorescence. Antibodies from clone 1A5G6 stained both the cytoplasm and the processes, and that from clone 1D3A3 stained only the cytoplasm and not the processes. 1A5G6 elicited no cross-reactivity towards human fetal and adult brain and lungs, liver, kidney or spleen, mouse neuroblastoma and melanoma, rat C<sub>6</sub> glioma, neuroblastoma × glioma hybrid and normal rat kidney cells. It gave 58–60% cross reactivity with the human neuroblastoma and T-cell leukemia cells. The antigenic component has been identified to be a membrane protein of molecular weight 25–30 kilodaltons by immunoblotting. Using C<sub>6</sub> glioma cells as antigenic stimulant 19 clones which were positive for C<sub>6</sub> glioma cells, but negative for rat liver cells as inferred by indirect immunofluorescence were selected. Antibodies secreted by all these gave positive reaction towards normal rat kidney and fetal rat kidney cells in culture. Distinct identity of these clones were ascertained by discernible staining patterns in indirect immunofluorescence on C<sub>6</sub> glioma cells.

**Keywords.** Brain tumors; monoclonal antibodies.

## Introduction

The vertebrate central nervous system is composed of diverse types of neuronal and glial cells. The latter are grouped into astrocytes, oligodendrocytes, ependymal cells and microglial cells. Many of the neuronal cell types have been identified by anatomists and physiologists on the basis of their distinctive arborization pattern and functional organization. A number of biochemical markers are now available which allow their unambiguous identification. For example, astrocytes contain large bundles of intermediate filaments, composed of vimentin and a 50 kilodalton protein called Glial fibrillary acidic protein (Raff *et al.*, 1983; Bjorklund *et al.*, 1984). Neurons contain the neurofilament protein (Steinert *et al.*, 1984) and galactocerebroside has been used as a surface marker for oligodendrocytes (Raff *et al.*, 1979).

Immunological approaches using antisera obtained by conventional procedures have been used in the past in the delineation of antigenic components in electrical and chemical synapses (Morita *et al.*, 1980; Nicoll *et al.*, 1980) and cell-adhesion molecules (Edelman, 1983). In view of heterogenous nature of polyclonal antibodies and complex antigenic stimulants often used these studies suffered from severe

---

\*To whom all correspondence should be addressed.

Abbreviations used: MEM, Eagle's Minimum Essential Medium; DMEM, Dulbecco-Vogt Modification of MEM; IMDM, Iscove's modification of DMEM; ELISA, Enzyme linked immunosorbent assay; PBS, Phosphate buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

to generate highly mono-specific antibodies against almost any antigenic component present in complex antigenic stimulants. A variety of complex antigenic stimulants ranging from fetal and adult brain tissue to clonal neuroblastoma and glioma cells maintained in culture have been used to generate panels of monoclonal antibodies eliciting varying degrees of specificity towards neuronal cells. They have been used to identify and characterise surface glycoproteins of the developing nervous system (Rougon *et al.*, 1983), the neural cell-adhesion molecules (Edelman, 1983), surface glycolipids of human neuroblastoma (Cheung *et al.*, 1985) and several surface markers of human gliomas (Wikstrand *et al.*, 1984). Recently a C<sub>6</sub> rat glioma tumor-associated surface protein antigen of molecular weight of 64 kilodaltons has been identified by a monoclonal antibody obtained by using whole C<sub>6</sub> cells as an antigenic stimulant (Luner and deVellis, 1986).

In earlier studies reported from this laboratory we have shown that a human glioblastoma multiforme cell line U87MG and a rat C<sub>6</sub> glioma cell line could be differentiated by the addition of dibutyryl cyclic AMP to the cultures (Gomathi, 1985; Sharma *et al.*, 1983). These cells thus serve as excellent model systems in the studies of tumor-associated and differentiation antigens. In this paper we report our observations on a panel of monoclonal antibodies generated against human glioblastoma multiforme and rat C<sub>6</sub> glioma cells as antigenic stimulants eliciting differential staining patterns in indirect immunofluorescence and varying range of cross reactivities towards cells of non-neuronal origin.

## Materials and methods

### Materials

Eagle's Minimum Essential Medium (MEM), Dulbecco-Vogt Modification of MEM (DMEM), Iscove's modification of DMEM (IMDM) and fetal bovine serum were obtained from Flow Laboratories, UK; immunoreagents from Cappel and Miles Laboratories, USA; polyethyleneglycol from Koch Light, USA; dimethylsulfoxide from Fisher Chemicals, USA. Sterile tissue culture ware and Elisa plates were obtained from Costar, USA. All other chemicals were from either Sigma Co., USA or British Drug House, Bombay.

### Cell lines

Human glioblastoma multiforme cell line U87MG, myeloma P3 × 63Ag8-653 and other cell lines tested for cross reactivity (table 1) were obtained from American Type Culture Collection, USA, and rat glioma C<sub>6</sub>Bu-1 was from Dr. Marshall Nirenberg, NIH, USA. Cells were grown in sterile 75 cm<sup>2</sup> flasks in appropriate culture media—MEM with 1 mM sodium pyruvate for U87MG and DMEM for C<sub>6</sub>Bu-1 containing 10% fetal bovine serum in the presence of penicillin (100 units/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 10% CO<sub>2</sub> in air at 37°C. Parental myelomas P3 × 63Ag8-653 and derived hybridomas were grown in IMDM supple-

**Table 1.** Reactivity of monoclonal antibodies 1A5G6, 1D3A3 and 2A4C12 with normal human tissues and cell lines by ELISA.

Cross reactivity %															
Cell lines										Normal human tissues					
SKNSH	JR4	FLF	NIH3T3	Neuro-2A	N <sub>18</sub> TG <sub>2</sub>	B <sub>16</sub> F <sub>10</sub>	NRK	C <sub>6</sub>	NxG Hyb	Brain (adult)	Brain (fetal)	Lung	Kidney	Spleen	Liver
1A5G6	60	58	9*	0	0	0	0	0	0	8*	9*	7*	0	0	4*
1D3A3	60	45	10*	0	0	0	0	0	0	3*	2*	20	34	12.5	40
2A4C12	100	95	90	97	99	100	100	100	86	85	90	85	90	90	95

SKNSH, human neuroblastoma; JR4, human T cell leukemia; FLF, human fetal lung fibroblast; NIH3T3, mouse fibroblast; Neuro-2A, mouse neuroblastoma; N<sub>18</sub>TG<sub>2</sub>, mouse neuroblastoma; B<sub>16</sub>F<sub>10</sub>, mouse melanoma; C<sub>6</sub>, rat glioma; NxG Hyb, mouse neuroblastoma x rat glioma hybrids.

\*Not significant.

Antibody reactivity with U87MG is taken as 100%.

mented with 10% fetal bovine serum and antibiotics in a humidified atmosphere as described before.

### *Normal human tissue*

The whole brain of a 12 weeks human fetus was obtained 1 h after prostaglandin-induced abortion. Adult brain and other normal tissues were obtained not later than 12 h after death from the mortuary of AIIMS. Membranes were prepared by the method of Ames and Nikaido (1976).

### *Immunization and cell fusion*

Balb/c mice (4–6 weeks old) were immunized with  $1 \times 10^6$  whole cells. The first injection (intraperitoneal) consisted of whole cells emulsified with Freund's complete adjuvant, followed by 3 weekly injections in Freund's incomplete adjuvant. The last injection of  $1 \times 10^6$  cells was given intravenously in saline. Three days later the spleen was removed aseptically, single cell suspension was prepared and fused with a non-secretory mouse myeloma cell line P3 $\times$ 63Ag8-653 in the presence of polyethyleneglycol 1000. The hybrids were plated in 96 well plates and screened for antibody production by an enzyme linked immunosorbent assay (ELISA).

### *ELISA*

$1 \times 10^5$  cells in 50  $\mu$ l of phosphate buffered saline (PBS) pH 7.4 or 25  $\mu$ g of membrane proteins were plated in the wells of PETG ELISA plates and incubated overnight at 37°C. After blocking the nonspecific binding sites with 2% bovine serum albumin in PBS pH 7.4 the wells were incubated for 1 h with 50  $\mu$ l of hybridoma supernatant at 37°C. The plates were rinsed several times with PBS and the wells incubated with second antibody-HRPO-conjugated rabbit antimouse (Fab)<sub>2</sub> diluted to 1:1000 in PBS for 1 h. After washing the wells with PBS the color was developed with 100  $\mu$ l of orthophenylene diamine (0.5 mg/ml in PBS) containing hydrogen peroxide (0.8  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub> in 1 ml of orthophenylene diamine. The reactions was terminated with 50  $\mu$ l of 5 N H<sub>2</sub>SO<sub>4</sub> and the optical density was read at 492 nm in an ELISA reader (Titertek Multiscan Flowlab).

### *Indirect immunofluorescence*

Cells were grown as monolayer on glass cover slips. They were washed with PBS and fixed with 4% formaldehyde in PBS, pH 7.4. The coverslips were incubated with hybridoma supernatant for 1 h at room temperature. Unbound antibodies were removed by washing 4 x with PBS and the cover slips were incubated with 1:40 dilution of FITC-conjugated rabbit antimouse antibody. After washing 4 x with PBS the coverslips were mounted in 90% glycerol in PBS and observed in a Nipkow

*electrophoresis and immunoblot*

100  $\mu$ g membrane proteins of U87MG cells were subjected to sodium dodecyl phosphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Ogita and Markert, 1991). Proteins separated on the gel were electrophoretically transferred to nitrocellulose sheets by the method of Towbin *et al.* (1979). The sheets were either stained with amido black or processed by ELISA with monoclonal antibodies.

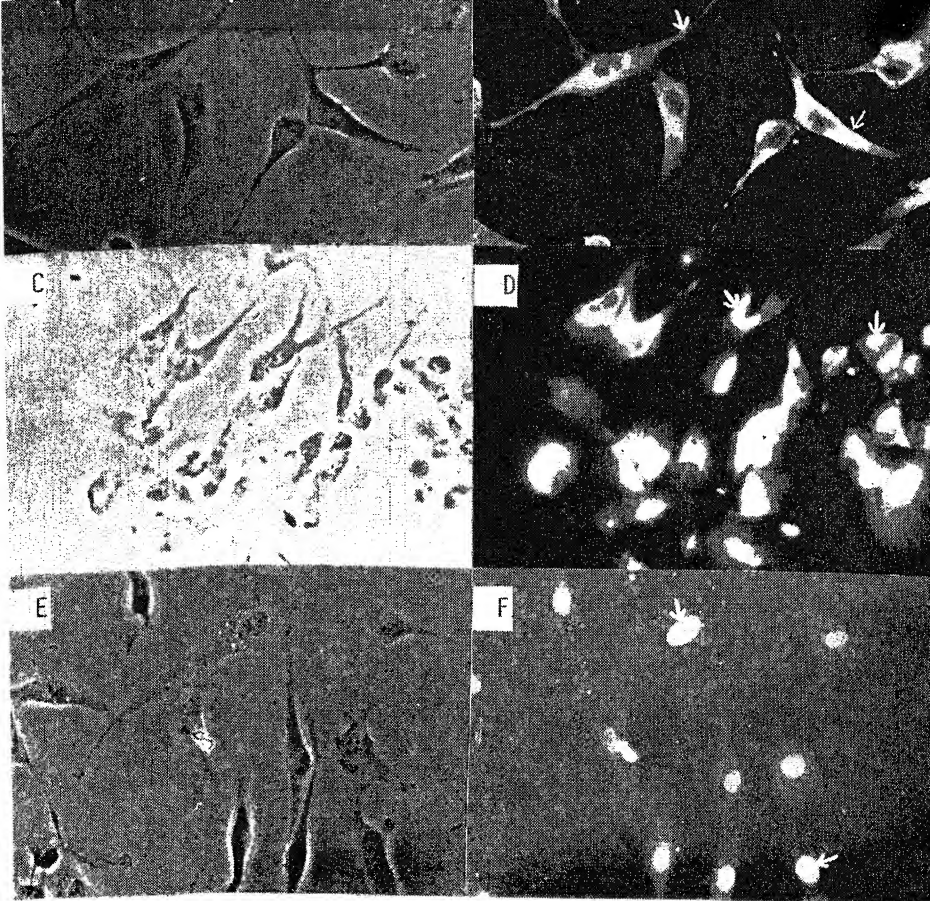
**Results and discussion**

There now exists an extensive literature on the use of monoclonal antibodies in the identification and characterization of cellular antigens (McKay *et al.*, 1981). The unprecedented resolution afforded by the mono-specific antibodies revealed the presence of many antigenic components unique to various cell types of diverse origin and different states of differentiation (Trisler *et al.*, 1981, 1983). At the same time many of these monospecific antibodies showed a wide range of affinities towards a variety of related or unrelated cell types. In our attempts towards a tentative classification of glioma cells of antibodies generated against human glioblastoma multiforme and C<sub>6</sub> glioma cells we have used two criteria: (i) spatial distribution of relevant antigens as ascertained by immunofluorescent studies and (ii) range of cross-reactivities with neuronal and non-neuronal cells of diverse origin as inferred by ELISA.

*Indirect immunofluorescence studies with monoclonal antibodies against U87MG human glioblastoma multiforme and rat C<sub>6</sub> glioma*

Antibodies secreted by 11 positive hybridoma clones (screened by ELISA) against U87MG whole cells as antigenic stimulants were examined by indirect immunofluorescence. The results are shown in figure 1. Their discernible staining patterns can be readily seen from the photomicrographs. (i) The antibody from clone 1A5G6 stained the cytoplasm and the processes, the fine processes are not uniformly stained and often show brightly stained spots (figure 1B). (ii) The antibody corresponding to clone 1D3A3 reveals highly asymmetric staining of cytoplasmic region (shown by arrow) with little or no staining of the fine processes (figure 1D). This can be readily ascertained by comparison with the phase contrast photomicrographs in figure 1A and 1C. (iii) The antibody secreted by clone 2A4C12 stains only the nuclei-both the cytoplasmic region and the processes remain completely unstained (figure 1F). Other remaining antibodies in the panel of 11 fall in one of the above 3 categories.

Photomicrographs included in figure 2 show antigenic distribution patterns as revealed by indirect immunofluorescence studies with representative antibodies from the panel of 19 hybridoma clones generated against rat C<sub>6</sub> glioma cells. Antibody 5 shows distinctive staining pattern with spotty appearance. The texture is indicative of localization of the antigenic components in discrete regions of the

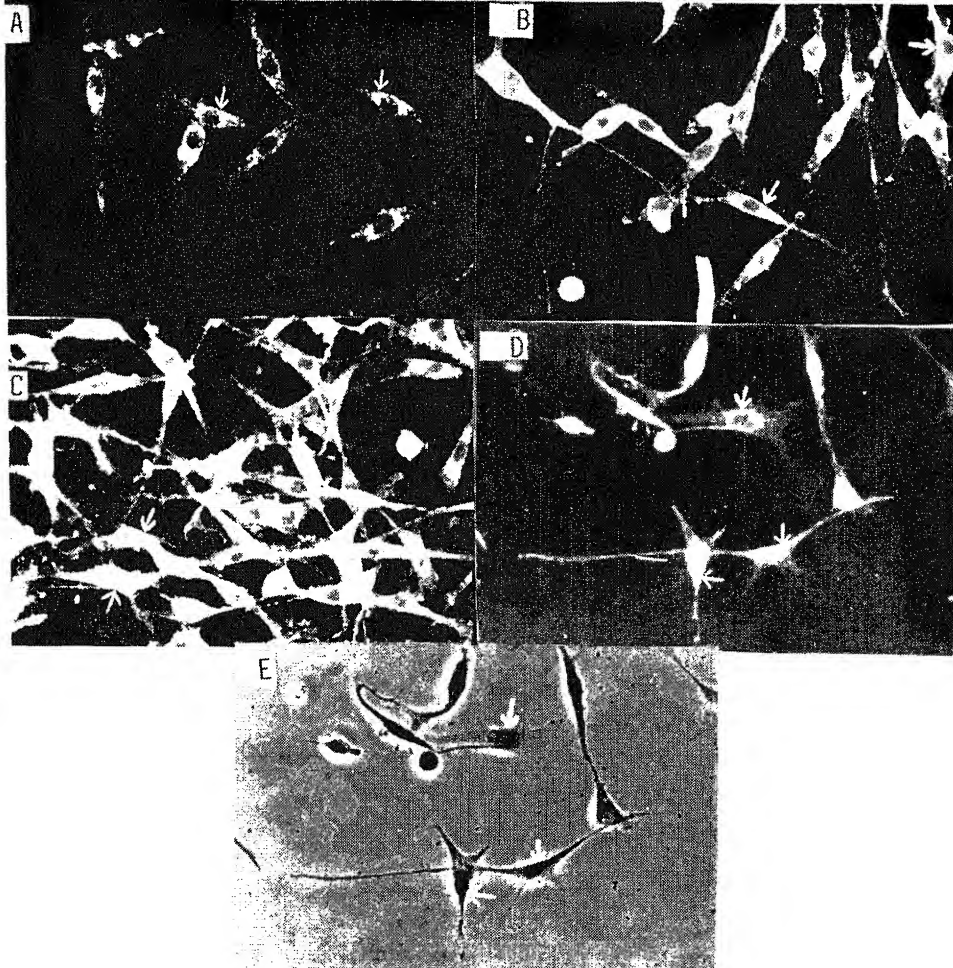


**Figure 1.** Indirect immunofluorescence staining of cellular antigens of U87MG cells by monoclonal antibodies. (B), Antibody 1A5G6; (D), antibody 1D3A3; (F), antibody 2A4C12. (A), (C) and (E) are the phase contrast photo-micrographs of the same field. Blanks with myeloma supernatant did not give any fluorescence ( $\times 200$ ).

discrete regions. Staining of the cytoplasmic region by antibody CE<sub>10</sub> is asymmetric and often more intense in the vicinity of nuclei as compared to that of the protoplasmic extrusion as shown in the corresponding phase contrast photograph (figure 2D,E).

#### *Range of cross reactivities displayed by the antibodies secreted by different clones*

In another type of investigations the cross reactivities of the antibodies with the membrane preparations from diverse types of cells of both neural and non-neural origin were examined by ELISA. The results for 3 clones 1A5G6, 1D3A3 and 2A4C12 secreting antibodies against human glioblastoma multiforme (U87MG) are sum-



**Figure 2.** Indirect immunofluorescence staining of cellular antigen of C<sub>6</sub> glioma cells by monoclonal antibodies. (A), (B), (C) and (D) are antibodies CE<sub>5</sub>, CE<sub>1</sub>, CE<sub>6</sub> and CE<sub>10</sub>. (E) is the phase contrast photomicrograph of the same field in CE<sub>10</sub>. Blanks with myeloma supernatant did not give any fluorescence ( $\times 200$ ).

marized in table 1. It is noticed from the data presented in table 1 that clone 1A5G6 shows no cross reactivity towards the cells of human origin including those from fetal and adult brain. It is also negative against mouse neuroblastoma or C<sub>6</sub> glioma cells. However, this clone shows significant levels of cross reactivity (58–60%) towards a human T cell leukemia and human neuroblastoma cells. Clones 1D3A3 and 2A4C12 on the other hand, recognize some widely distributed antigenic components present in a variety of cell types.

Cross reactivities of the clones secreting antibodies against rat C<sub>6</sub> glioma are summarized in table 2. All the clones tested exhibited 60–90% cross reactivity with normal rat kidney (NRK) cells. The fluorescence pattern revealed by the antibody



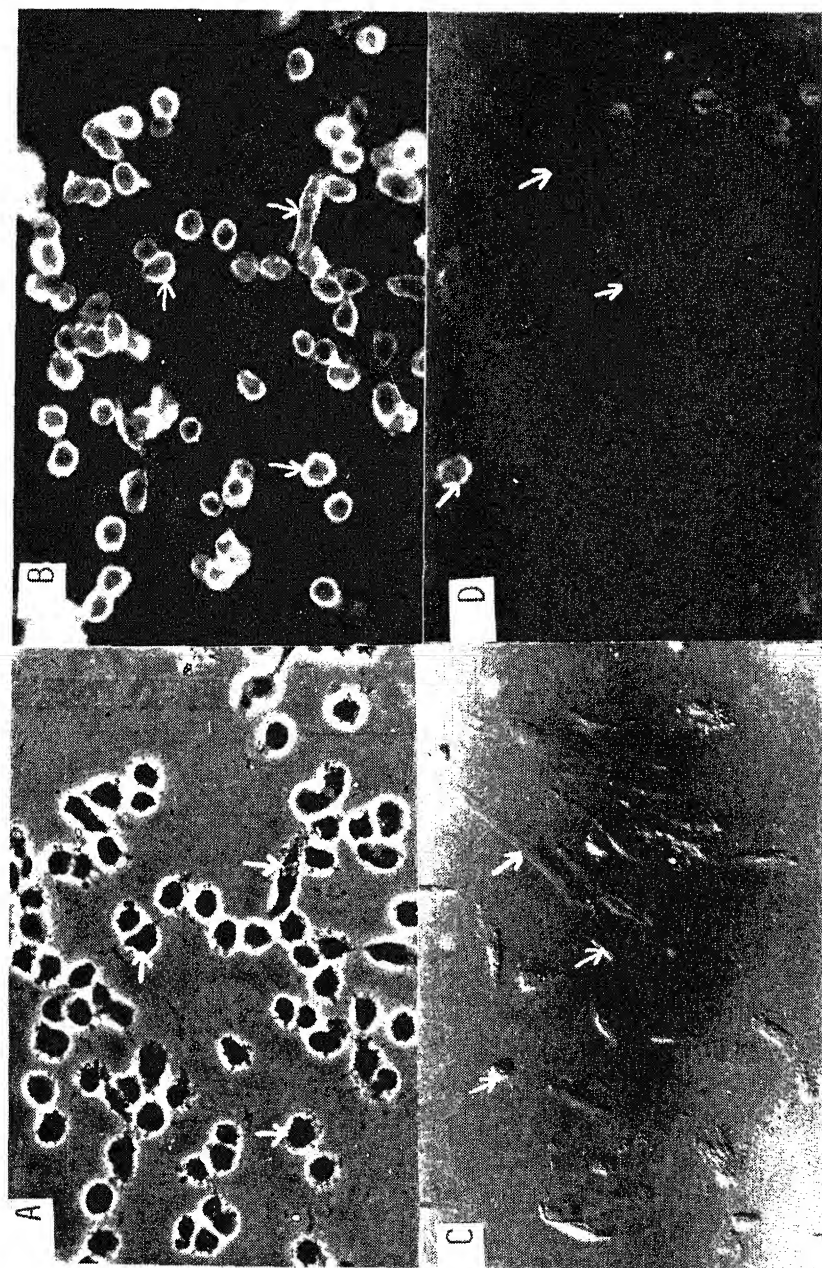
Table 2. Reactivity of monoclonal antibodies CE<sub>1</sub>, CE<sub>5</sub>, CE<sub>6</sub> and CE<sub>10</sub> with normal rat tissues and cell lines by ELISA.

		Cross reactivity (%)									
		Cell lines				Normal rat tissues					
		NIH3T3	Neuro-2A	NRK	NxG Hyb	Brain (adult)	Brain (fetal)	Lung	Kidney	Intestine	Pancreas Adrenal
CE <sub>1</sub>	25	26	85	7*	9*	8*	8*	19	9*	7*	3* 8*
CE <sub>5</sub>	0*	17	73	8*	10*	8*	8*	9	16	22	25 4*
CE <sub>6</sub>	3*	18	62	15	9*	14	14	19	14	31	21 5*
CE <sub>10</sub>	6*	2	65	21	10*	14	14	22	12	29	15 10*

Cell lines and tissues which did not cross react were, N<sub>18</sub>TG<sub>2</sub>; U87MG, human glioblastoma multiforme; SKNSH, SKNMC and IMR32, human neuroblastoma; SKMEL, human melanoma; JR4, and normal rat-heart; Spleen: testis; ovary; muscle and liver.

\*Not significant.

Antibody reactivity with C<sub>6</sub> glioma is taken as 100%.



**Figure 3.** Indirect immunofluorescence staining of cellular antigens of NRK and primary cultures of fetal rat kidney by antibody CE<sub>10</sub>. (A) and (B), NRK cells; (C) and (D), fetal rat kidney. (A) and (C) are the phase contrast photomicrographs of the fields represented in (B) and (D).

rescence, with the cytoplasm unstained (Figure 3B). Similar ring-like fluorescence on the epithelial cells was seen on the primary cultures of fetal rat kidney cells (figure 3D). It is interesting to note that fibroblasts in the same field (figure 3C) were unstained.

The studies presented in this paper essentially comprise preliminary observations on the panels of monoclonal antibodies generated against U87MG and C<sub>6</sub> glioma cells. Immunocytochemical investigations and range of cross-reactivities of the antibodies with different tissues and cell lines have enabled us to tentatively classify them into distinct groups.

The clone 1A5G6 is particularly interesting as it shows a very restrictive range of cross reactivity. Membrane proteins from U87MG cells were separated by SDS-PAGE and transferred electrophoretically (Western blot) onto nitrocellulose sheet. Antigenic component corresponding to the clone 1A5G6 was identified as a 25–30 kilodalton protein by ELISA (Tsang *et al.*, 1983) using horse radish peroxidase conjugated second antibody (figure 4).

Concerning the clones secreting antibodies against C<sub>6</sub> glioma, it is indeed surprising that all of them elicit strong cross-reactivity towards NRK cells as well as fetal rat kidney cells in culture. That the relevant antigenic components are localized on

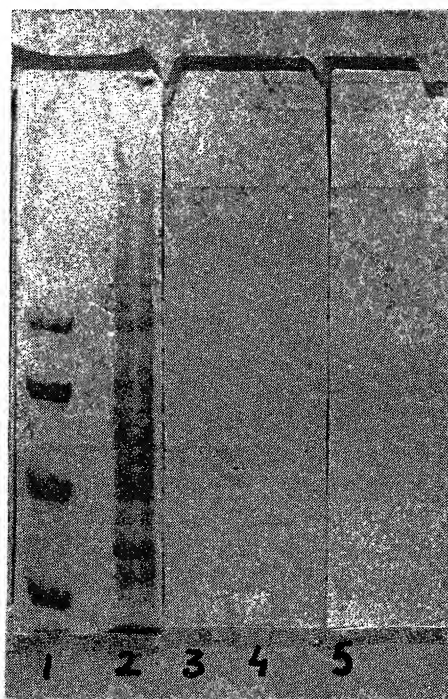


Figure 4. Identification of the U87MG membrane antigens by antibody 1A5G6. Lane 1, standard molecular weight markers. Lane 2, surface proteins of U87MG cells electrophore-

the specific cell types is apparent from the fact that the neuroblast cells present in primary rat kidney culture (figure 3C,D) remain completely unstained. It is not clear at present as to whether sharing of a number of antigenic components by rat kidney cells and C<sub>6</sub> glioma cells has some ontogenetic basis. Work on the molecular characterization of various antigenic components, which may throw light on this question is in progress.

## acknowledgements

This work was supported by financial assistance to SKS from Department of Science and Technology, New Delhi (No. 12(1)/80-SERC.STP II). We are thankful to Prof. N. Singh, Delhi University South Campus for helpful discussions in the preparation of this manuscript. E.S. is the recipient of Research Fellowship from the Indian Council of Medical Research, New Delhi.

## References

- Arnesen, G. F. L. and Nikaido, K. (1976) *Biochemistry*, **15**, 616.
- Arvidsson, H., Dahl, D., Olson, L. and Seiger, A. (1984) *J. Neurosci.*, **4**, 978.
- Beckh, N. K.V., Saarinen, U. M., Neely, J. E., Landmeier, B., Donovan, D. and Coccia, P. F. (1985) *Cancer Res.*, **45**, 2642.
- Blumberg, G. M. (1983) *Science*, **219**, 450.
- Chatterjee, K. G. (1985) *Analysis of the membrane glycoproteins of the human glioblastoma cell line U87MG*, M.Sc. thesis, All India Institute of Medical Sciences, New Delhi.
- Chen, G. and Milstein, C. (1975) *Nature (London)*, **256**, 495.
- Cohen, S. J. and deVellis, J. (1986) *Cancer Res.*, **46**, 863.
- Cohen, R., Raff, M. C. and Reichardt, L. F. (1981) in *Monoclonal Antibodies to Neural Antigens* (Cold Spring Harbor Reports in NeuroSci.) p. 1.
- Cosans, K., North, R. A. and Katayama, Y. (1980) *Nature (London)*, **287**, 151.
- Croll, R. A., Schenker, C. and Leeman, S. E. (1980) *Annu. Rev. Biochem.*, **3**, 227.
- Czernik, Z. and Markert, C. L. (1979) *Anal. Biochem.*, **99**, 233.
- Feldman, M. C., Fields, K. L., Hakomori, S., Mirsky, R., Pruss, R. M. and Winter, J. (1979) *Brain Res.*, **174**, 283.
- Feldman, M. C., Miller, R. H. and Nobel, M. (1983) *Cold Spring Harb Symp.*, **68**, 569.
- Ferguson, G., Hirsch, M. R., Gruenert, J. L. and Goridis, C. (1983) *Neuroscience*, **10**, 511.
- Garg, S. K., Kumar, R. and Singh, U. N. (1983) in *Monoclonal Antibodies and Cancer* (eds B. D. Boss, J. Langman, I. Trowbridge and R. Dulbecco) (Florida: Acad. Press) p. 239.
- Ginsberg, P. M., Jones, J. C. R. and Goldman, R. D. (1984) *J. Cell Biol.*, **99**, 22.
- Ginsberg, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350.
- Ginsberg, G. D., Schneider, M. D. and Nirenberg, M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2145.
- Ginsberg, G. D., Schneider, M. D., Moskal, J. R. and Nirenberg, M. (1983) *Cold Spring Harb. Rep. Neurosci.*, **1**, 231.
- Ginsberg, V. C. W., Peralta, J. M. and Simons, R. A. (1983) *Methods Enzymol.*, **92**, 377.
- Goldman, C. J., Bigner, S. H. and Bigner, D. D. (1984) *J. Neuroimmunol.*, **6**, 169.



## Scavenging of superoxide radical by ascorbic acid

ANURADHA NANDI and I. B. CHATTERJEE\*

Department of Biochemistry, University College of Science, 35, Ballygunge Circular Road, Calcutta 700 019, India

**Abstract.** Using acetaldehyde and xanthine oxidase as the source of superoxide radical, the second order rate constant for the reaction between ascorbic acid and superoxide radical was estimated to be  $8.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . In rats, the average tissue concentration of ascorbic acid was of the order of  $10^{-3} \text{ M}$  and that of superoxide dismutase was of the order of  $10^{-6} \text{ M}$ . So, taking together both the rate constants and the tissue concentrations, the efficacy of ascorbic acid for scavenging superoxide radical in animal tissues appears to be better than that of superoxide dismutase. The significance of ascorbic acid as a scavenger of superoxide radical has been discussed from the point of view of the evolution of ascorbic acid synthesizing capacity of terrestrial vertebrates.

**Keywords.** Superoxide radical; ascorbic acid; superoxide dismutase; rate constant.

### Introduction

The potentiality of ascorbic acid as a scavenger of superoxide radical ( $\text{O}_2^-$ ) has earlier been discussed by Nishikimi (1975). He has found the second order rate constant for the reaction between ascorbic acid and  $\text{O}_2^-$ , generated by xanthine-xanthine oxidase system, to be  $2.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ . He has followed the rate of oxidation of ascorbic acid with  $\text{O}_2^-$  at a wave length of 249.6 nm which is away from the absorption maximum of ascorbic acid at 265 nm. Moreover, when xanthine oxidase is added to a system containing xanthine and ascorbic acid about 23% of the decrease in absorbance at 265 nm (apparently taken as the oxidation of ascorbic acid) is contributed by the oxidation of xanthine to uric acid. Furthermore, uric acid is also a scavenger of  $\text{O}_2^-$  (Ames *et al.*, 1981; Kellogg and Fridovich, 1977). Previously, we determined the rate constant ( $K_{\text{AH}_2}$ ) for the reaction between  $\text{O}_2^-$  and ascorbic acid using a combination of titrimetric and photometric methods. The value was found to be  $5.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (Som *et al.*, 1983). Considering that determination of the rate of oxidation of very small concentrations of ascorbic acid by titrimetric method might be subjected to significant error, we have reevaluated the rate constant for the reaction between  $\text{O}_2^-$  and ascorbic acid using acetaldehyde instead of xanthine as a substrate for xanthine oxidase. The reaction between acetaldehyde and xanthine oxidase as a source of  $\text{O}_2^-$  has been effectively used by Kellogg and Fridovich (1977). The results are presented in this communication.

### Materials and methods

found to be free from superoxide dismutase contamination. Acetaldehyde was purchased from British Drug House, Bombay and ascorbic acid was a product of Sarabhai M Chemicals.

The activity of superoxide dismutase was assayed by its ability to inhibit the reduction of cytochrome C by the xanthine-xanthine oxidase system (McCord and Fridovich, 1969). The molar concentration of superoxide dismutase was determined from its activity; one unit of superoxide dismutase was taken to be  $3.1 \times 10^{-9}$  M (McCord and Fridovich, 1969).

The assay mixture for the reaction between ascorbic acid and  $O_2^-$  generated by acetaldehyde-xanthine oxidase system contained 50 mM air-equilibrated potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 25 mM acetaldehyde, 40  $\mu$ g catalase and graded concentrations of ascorbic acid in a final volume of 2 ml. The reaction was started by addition of 7 mU of xanthine oxidase and oxidation of ascorbic acid was followed by change of absorbance at 265 nm in a 1.0 cm cuvette using a Hitachi Model 200-20 spectrophotometer with recorder. After an equilibration period of 30 s, readings were taken for 2 min. Under the conditions, there was no spontaneous oxidation of ascorbic acid in the absence of  $O_2^-$ . Also no change of absorbance at 265 nm was observed with acetaldehyde and ascorbic acid, acetaldehyde and xanthine oxidase or with ascorbic acid and xanthine oxidase. The optimum concentration of ascorbic acid at which the rate of its oxidation with  $O_2^-$  generated by acetaldehyde and xanthine oxidase system showed saturation was determined. In a separate set of experiment, using this optimum concentration of ascorbic acid the rate of oxidation was determined in the presence of various concentrations of superoxide dismutase.

Superoxide dismutase activity in tissue homogenate was determined by xanthine-xanthine oxidase system (Crapo *et al.*, 1978). Tissue homogenate (1:9 for lung and 1:4 for other tissues) was prepared in ice cold 0.25 M sucrose containing 0.5% Triton X-100. The crude homogenate was centrifuged at 17,000 *g* for 30 min and the supernatant was dialyzed against 25 mM potassium phosphate buffer, pH 7.8 and assayed for superoxide dismutase activity as described below. Erythrocyte superoxide dismutase activity was assayed after removing the haemoglobin by Tsuchihashi procedure (Crapo *et al.*, 1978). This was reported as the blood superoxide dismutase content in table 1. Plasma does not have any superoxide dismutase activity. The assay mixture contained  $4 \times 10^{-5}$  M xanthine, 2 mM EDTA,  $3 \times 10^{-5}$  M cytochrome C, 40  $\mu$ g catalase, 10  $\mu$ M KCN, required amount of tissue homogenate and 0.1 M air-equilibrated potassium phosphate buffer pH 7.8 in a final volume of 2 ml. The reaction was initiated by addition of 7 mU of xanthine oxidase. The increase of absorbance was measured at 550 nm for 2 min after an equilibration period of 30 s. Under the condition, the rate of change in absorbance in the absence of tissue homogenate was approximately 0.02 per min. One unit of superoxide dismutase is described as the amount of enzyme required to cause 50% inhibition of ferricytochrome C reduction per 3 ml of assay mixture. For different tissues, the amount of tissue homogenate added to the assay mixture was so adjusted that approximately 50% inhibition was observed in each case.

Ascorbic acid in different tissues was estimated as 2:4 dinitrophenylhydrazine derivative following the method of Chatterjee and Banerjee (1979).

The rate of disappearance of  $O_2^-$  ( $V_{AH_2}$ ) in the presence of graded concentrations of ascorbic acid and xanthine oxidase is shown in figure 1. The saturating level of ascorbic acid was found to be  $5.75 \times 10^{-6}$  M.

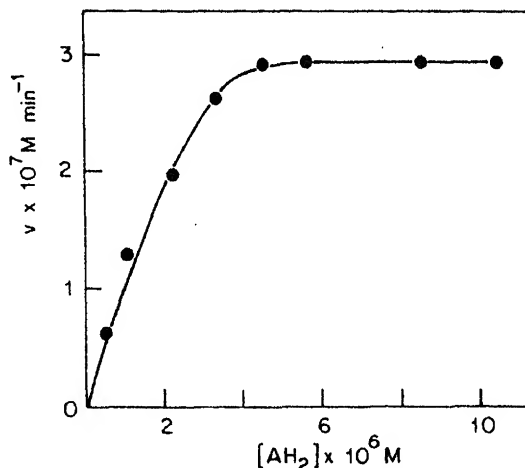


Figure 1. The rate of oxidation of ascorbic acid as a function of its concentration. Details of the method are given in the text.

*Effect of varied concentrations of superoxide dismutase on the oxidation of ascorbic acid by  $O_2^-$*

Taking the saturating concentration of ascorbic acid i.e.  $5.75 \times 10^{-6}$  M and keeping other conditions constant, the effect of varied concentrations of superoxide dismutase on the rate of oxidation of ascorbic acid was examined and data were plotted in figure 2.

*Rate constant ( $K_{AH_2}$ ) of the reaction between ascorbic acid and  $O_2^-$*

The reaction of  $O_2^-$  with ascorbic acid ( $AH_2$ ) can be represented in the following way:

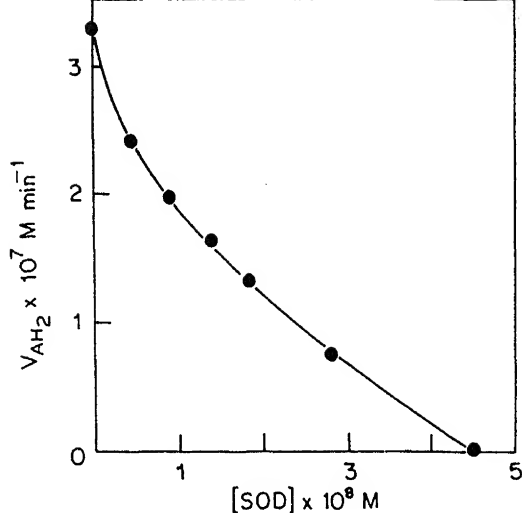


Where  $AH\cdot$  is the ascorbate free radical. Therefore, the rate of disappearance of  $O_2^-$  ( $V_{AH_2}$ ) can be formulated as

$$V_{AH_2} = K_{AH_2} [AH_2] [O_2^-] \quad (2)$$

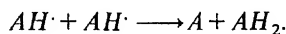
$AH\cdot$  is a relatively non-reactive species (Bielski *et al.*, 1975) and it decays mainly by





**Figure 2.** Inhibitory effect of superoxide dismutase on the rate of oxidation of ascorbic acid by  $O_2^{\cdot -}$ . The saturating concentration of ascorbic acid used was  $5.75 \times 10^{-6}$  M. Details of the method are given in the text.

disproportionation. A represents dehydroascorbic acid.



On the other hand, the reaction between  $O_2^{\cdot -}$  and superoxide dismutase is as follows:



This enzyme-catalyzed reaction is first order with respect to enzyme and to  $O_2^{\cdot -}$  (Rotilio *et al.*, 1972; Klug-Roth *et al.*, 1972) and the rate is given by

$$V_{SOD} = K_{SOD}[SOD][O_2^{\cdot -}]. \quad (4)$$

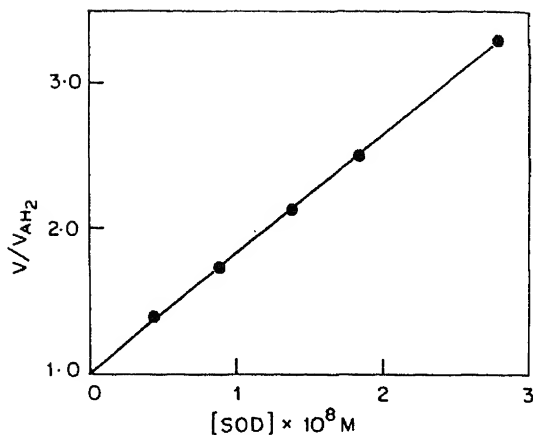
Under the experimental conditions used, at a saturating concentration of ascorbic acid and in the presence of superoxide dismutase,  $O_2^{\cdot -}$  disappears through both reactions (1) and (3). Under such a condition at a steady state, the rate of generation of  $O_2^{\cdot -}$  ( $V$ ) by the reaction of acetaldehyde and xanthine oxidase is the sum of the rates of disappearance of  $O_2^{\cdot -}$  through reactions (1) and (3).

$$\text{i.e. } V = V_{AH_2} + V_{SOD}. \quad (5)$$

Then the following equation can be derived from equations (2), (4) and (5):

$$\frac{V}{V_{AH_2}} = 1 + \frac{K_{SOD}[SOD]}{K_{AH_2}[AH_2]}. \quad (6)$$

Using the saturating level of ascorbic acid, the effect of varied concentrations of superoxide dismutase on the rate of oxidation of ascorbic acid was examined and data were plotted according to equation (6). Figure 3 shows that a straight line



**Figure 3.** Relationship between  $V/V_{AH_2}$  and concentration of superoxide dismutase. Details of the method are given in the text.

$10^9 \text{ M}^{-1} \text{ s}^{-1}$  (Rotilio *et al.*, 1972), the rate constant  $K_{AH_2}$  for the reaction between ascorbic acid and  $O_2^-$  was calculated from the slope of the graph to be  $8.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ .

#### *Tissue concentrations of superoxide dismutase and ascorbic acid of rat*

The levels of ascorbic acid and total superoxide dismutase in different tissues of rat are given in table 1. The data indicate that the average concentrations of superoxide dismutase in rat tissues is about 1000 times less than that of ascorbic acid.

**Table 1.** Levels of total superoxide dismutase and ascorbic acid in rat.

Tissue	Superoxide dismutase $\text{M} \times 10^6$	Ascorbic acid $\text{M} \times 10^3$
Blood	$14 \pm 0.20$	—
Plasma	—	$0.04 \pm 0.006$
Liver	$2.60 \pm 0.14$	$1.40 \pm 0.110$
Adrenal glands	$2.60 \pm 0.38$	$6.80 \pm 0.700$
Heart	$1.40 \pm 0.10$	$0.57 \pm 0.060$
Brain	$0.80 \pm 0.07$	$1.60 \pm 0.140$
Kidney	$0.69 \pm 0.08$	$0.79 \pm 0.040$
Lung	$0.55 \pm 0.08$	$1.20 \pm 0.100$
Pancreas	$0.46 \pm 0.06$	$0.89 \pm 0.070$

Each result represents an average ( $\pm$ SD) from 8 young male albino rats (Charles Foster strain) of body weight between 125–175 g. Details of methods are given in the text.

## **Discussion**

tions of collagen prolyl hydroxylase require molecular oxygen,  $\text{Fe}^{2+}$  ions, and  $\alpha$ -ketoglutarate for the activity. The requirement of ascorbic acid is not absolute (Barnes, 1975). Addition of ascorbic acid stimulates the hydroxylation, but ascorbic acid may be replaced by tetrahydropteridine, dithiothreitol, catalase or albumin (Ramachandran and Reddi, 1976). Thus, the role of ascorbic acid is difficult to explain, particularly because solutions of ascorbate,  $\text{Fe}^{2+}$  ions, and oxygen are known to generate hydroxyl radicals non-enzymatically which can participate in hydroxylating reactions (Bade and Gould, 1969).

We consider that the biological function of ascorbic acid may well be explained from the view point of evolution of the ascorbic acid synthesizing capacity in terrestrial vertebrates. The capacity to synthesize ascorbic acid is absent in fishes and it appears in the amphibians (Chatterjee, 1973; Roy Chaudhuri and Chatterjee, 1969; Chatterjee *et al.*, 1975). The average oxygen concentration of water is 0.3%, whereas that of air in the time of evolution of amphibians (about 350 million years ago) was about 10% (Cloud and Gibor, 1970). This means that during evolution from aquatic to the terrestrial mode of life the amphibians had to face an oxygen concentration 30 times that of fishes. One would conceive that such a tremendous increase in oxygen concentration would have been extremely toxic and fatal, unless the newly evolved tetrapods acquired a strong defence mechanism against oxygen toxicity. It is now known that the form of oxygen responsible for oxygen toxicity is  $\text{O}_2^-$ , which may act secondarily through hydroxyl radical. In microorganisms, superoxide dismutase increased markedly (15–20 times) with elevation of oxygen concentration, apparently to survive against oxygen toxicity (Fridovich, 1974). One would, therefore, expect that like that observed in the microorganisms, the superoxide dismutase should have adaptively increased many-fold in amphibians. However, we observed that superoxide dismutase activity of liver of amphibians did not significantly increase over that of fishes (Som *et al.*, 1983). Incidentally, the amphibians started synthesizing ascorbic acid, which is also a potential scavenger of  $\text{O}_2^-$  (Nishikimi, 1975; Som *et al.*, 1983; Sawyer *et al.*, 1985). The second order rate constant  $K_{AH_2}$  for the reaction between  $\text{O}_2^-$  and ascorbic acid at pH 7.0 has been found to be  $8.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . Although the rate constant  $K_{SOD}$  between  $\text{O}_2^-$  and superoxide dismutase is  $1.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , which is approximately 23 times more than that of  $K_{AH_2}$ , the average concentration of superoxide dismutase in tissues of rat is in the order of  $10^{-6} \text{ M}$  which is about 1000 times less than that of the average tissue concentration of ascorbic acid. It would thus appear that taking together the rate constants and the tissue concentrations, the efficacy of ascorbic acid for scavenging  $\text{O}_2^-$  in animal tissues is better than that of superoxide dismutase. There is a further advantage of ascorbic acid as a scavenger of  $\text{O}_2^-$ . The product of reaction between ascorbic acid and  $\text{O}_2^-$  is dehydroascorbic acid. In animal tissues, dehydroascorbic acid is reduced back to ascorbic acid by glutathione resulting in recycling of ascorbic acid (Basu *et al.*, 1979; Som *et al.*, 1980). We have indicated before that the scavenging of  $\text{O}_2^-$  by ascorbic acid in conjunction with glutathione is more economical (Som *et al.*, 1983). We consider that the evolution of vertebrates from aquatic medium to the terrestrial atmosphere was contingent on the evolution of a

new enzyme, namely L-gulonolactone oxidase, to provide the newly evolved tetrapods with adequate amount of ascorbic acid, a chemical defence against oxygen toxicity. Apparently, this might have happened to compensate the lack of adaptive increase of the enzymatic defence, namely superoxide dismutase, in the early tetrapods, so that evolution of terrestrial vertebrates could continue.

## Acknowledgements

This work was supported by grants-in-aid from the Indian Council of Medical Research and the University Grants Commission, New Delhi.

## References

- Ames, B. N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6858.
- Bade, M. L. and Gould, B. S. (1969) *FEBS lett.*, **4**, 200.
- Barnes, M. J. (1975) *Ann. N.Y. Acad. Sci.*, **258**, 264.
- Basu, S., Som, S., Deb, S., Mukherjee, D. and Chatterjee, I. B. (1979) *Biochem. Biophys. Res. Commun.*, **90**, 1335.
- Bielski, B. H. J., Richter, H. W. and Chan, P. C. (1975) **258**, 231.
- Chatterjee, I. B. and Banerjee, A. (1979) *Anal. Biochem.*, **98**, 368.
- Chatterjee, I. B. (1978) in *World Review of Nutrition and Dietetics*, (Basel, Switzerland: S. Karger), **30**, 69.
- Chatterjee, I. B. (1973) *Science*, **182**, 1271.
- Chatterjee, I. B. (1973) *Sci. Cult.*, **39**, 210.
- Chatterjee, I. B., Majumdar, A. K., Nandi, B. K. and Subramanian, N. (1975) *Ann. N.Y. Acad. Sci.*, **258**, 24.
- Cloud, P. and Gibor, A. (1970) *Sci. Am.*, **223**, 110.
- Crapo, J. D., McCord, J. M. and Fridovich, I. (1978) *Methods Enzymol.*, **53**, 382.
- Fridovich, I. (1974) *Adv. Enzymol.*, **41**, 35.
- Gallop, P. M., Blumenfeld, O. O. and Seifter, S. (1972) *Annu. Rev. Biochem.*, **41**, 617.
- Kellogg, E. W. Tert. and Fridovich, I. (1977) *J. Biol. Chem.*, **252**, 6721.
- Klug-Roth, D., Fridovich, I. and Rabani, J. (1972) *J. Am. Chem. Soc.*, **95**, 2786.
- McCord, J. M. and Fridovich, I. (1969) *J. Biol. Chem.*, **244**, 6049.
- Nishikimi, M. (1975) *Biochem. Biophys. Res. Commun.*, **63**, 463.
- Ramachandran, G. N. and Reddi, A. H. (1976) *Biochemistry of collagen* (New York: Plenum Press) p. 174.
- Rotilio, G., Bray, R. C. and Fielden, M. (1972) *Biochim. Biophys. Acta*, **268**, 605.
- Roy Chaudhuri, C. and Chatterjee, I. B. (1969) *Science*, **164**, 435.
- Sawyer, D. T., Calderwood, T. S., Johlman, C. L. and Wilkins, C. L. (1985) *J. Org. Chem.*, **50**, 1409.
- Som, S., Basu, S., Deb, S., Mukherjee, D. and Chatterjee, I. B. (1980) *Curr. Sci.*, **49**, 195.
- Som, S., Raha, C. and Chatterjee, I. B. (1983) *Acta Vitaminol. Enzymol.*, **5**, 243.



# Alteration of the acyl chain composition of free fatty acids, acyl coenzyme A and other lipids by dietary polyunsaturated fats

M. RENUKA PRASAD\*, BRENDA CULP\*\* and WILLIAM E. M. LANDS†

Department of Biological Chemistry, University of Illinois at Chicago; 1853 W. Polk St., Chicago, Illinois 60612, USA

\*Present address: Department of Surgery, University of Connecticut Health Center, Farmington, Connecticut 06032, USA

\*\*Present address: 11201 Fetterly Road West, Minnetonka, Minnesota 55343, USA

**Abstract.** Dietary alterations were used to demonstrate selective handling of fatty acids during their redistribution *in vivo*. Differences in the mol per cent of individual acyl chains in the non-esterified fatty acid, acyl-coenzyme A and phospholipid fractions reflected a result of relative precursor abundance combined with enzymic selectivities. Selective distributions were observed in the utilization of individual acyl chains between 16:0 and 18:0, 18:1 and 18:2, and among 20:3, 20:4 and 20:5, 22:6 by ligase(s), hydrolase(s) and acyl-transferases.

The variations in the mol per cent of linoleate present in the acyl-coenzyme A fraction of liver relative to that in the non-esterified fatty acids suggested an *in vivo* regulation of the level of linoleoyl-coenzyme A that influenced the synthesis of both arachidonoyl-coenzyme A and lipids.

The greater abundance of eicosapentaenoic acid in the free fatty acid fraction relative to that in the acyl-coenzyme A fraction may increase the ability of dietary 20:5n-3 to be an effective inhibitor of the synthesis of prostaglandins derived from 20:4n-6.

**Keywords.** Acyl-coenzyme A; polyunsaturated fatty acids; lipids; non-esterified fatty acids.

## Introduction

It has been well established that a highly selective *in vivo* handling of fatty acids results in saturated fatty acids being placed predominantly in position 1 of glycerolipids in most tissues whereas unsaturated acids occupy position 2 (Hill and Lands, 1970; Lands and Crawford, 1975). Some selective recognition of acyl chains occurs in reactions catalyzed by liver acid: coenzyme A (CoA) ligases (Groot *et al.*, 1976; Marcel and Suzue, 1972; Lands, 1979), acyl-CoA hydrolases (Ellingson *et al.*, 1970; Jezyk and Hughes, 1971) and acyl-CoA acyltransferases (Lands and Crawford, 1975; Lands, 1979; Yamada and Okuyama, 1978; Holub, 1976; Lands *et al.*, 1982). However, until the recent work in the laboratory of Majerus (Neufeld *et al.*, 1983), little selectivity among the long-chain fatty acids had been reported for long-chain acid:CoA ligases (Hill and Lands, 1970; Groot *et al.*, 1976; Lands, 1979). Now the recognition of an arachidonate-selective ligase that can be separated from the non-specific ligase (Laposta *et al.*, 1985) opens new approaches to study the regulation of acyl chain flux. Interestingly the non-specific activity may be dominant only in liver and adipose tissue, two tissues that have major roles in general (non-selective) lipid mobilization and turnover. The acyl-CoA hydrolases apparently can discriminate between the CoA-esters of palmitate and stearate and among the CoA-esters of

phosphatases, like acyl-CoA:glycerophosphate acyltransferase, acyl-CoA:1-acyl glycerol phosphate acyltransferase and acyl-CoA:phospholipid acyltransferase have high selectivities that depend upon small structural details of the fatty acids (Lands, 1979). Studies on the specificity of acyltransferases have given significant amounts of information to help understand the capacity of these enzymes to control the positional placement of mono-, di- and tetra-unsaturated fatty acids in the glycerolipids. However, the accumulated *in vitro* data could not predict accurately the acyl chain composition of free fatty acids, acyl-CoA, phospholipids and triglycerides of rat liver under various dietary conditions because the actual composition of the precursor pools was unknown.

To help interpret the selectivities that occur *in vivo*, we examined the transient accumulation of polyunsaturated fatty acids in several related lipid fractions in liver. For this purpose we chose two lipid supplements: fish oil (rich in the long-chain n-3 polyunsaturated acids) and corn oil (rich in 18:2n-6). These supplements were added to either fat-free diet or to a regular chow diet. The acyl chain compositions of the various fractions of liver lipids were used to evaluate the extent of selective recognition of acyl chains *in vivo* by enzymes of fatty acid metabolism in liver.

## Materials and methods

Sodium borohydride, malate dehydrogenase, citrate synthetase, phosphotransacetylase, NAD<sup>+</sup>, CoA, L-maleic Acid and dithiothreitol were all obtained from Sigma Chemical Co., St. Louis, Missouri, USA. The t-butyldimethylchlorosilane-imidazole reagent was purchased from Applied Science, State College, Pennsylvania, USA. Neutral alumina and silica gel H were from E. Merck, Darmstadt, FRG. Fatty alcohols and acids were obtained from Nuchek Prep, Elysian, Minnesota, USA. All solvents were reagent grade and distilled before use. Acyl-CoA esters used for reference standards were prepared as described by Okuyama *et al.* (1969).

### *Sucrose-casein diets*

Sprague-Dawley male weanling rats (3 days after weaning) were fed *ad libitum* for 50 days a pelleted fat-free diet based on sucrose and casein (ICN Nutritional Biochemicals, Cleveland, Ohio). Then the rats were divided into 3 groups. One group continued with the fat-free diet, and the diet of the other two groups was supplemented with 13% (v/w) of either menhaden fish oil (kindly provided by Dr. A. P. Bimbo, Zapata Haynie Corp.; Reedville, Virginia) or commercial corn oil. The oil supplements were prepared fresh every 3 days by thorough mixing of measured amounts of the oils with the food pellets. Each oil contained 0.02% t-butylhydroquinone as an added antioxidant and represented about 20% of the total calories consumed.

### *Chow-based diets*

Additional rats had been raised on a regular chow diet until they were about 2 months old and weighed 130–160 g. The chow diet was then supplemented for 14 days

Where indicated, the animals were fasted 18 h before being anesthetized with sodium phenobarbital (50 mg i.p. per kg body wt.). Their abdomen was surgically opened, and the liver was quickly removed, rinsed with ice-cold saline (0.9%, w/v) to remove blood, and then frozen at  $-70^{\circ}\text{C}$  (dry ice-acetone bath). The frozen livers were lyophilized to obtain the dry tissue for more convenient extraction of the lipids.

#### *Analysis of acyl-CoA, fatty acids, phospholipids and triacylglycerols*

The overall amount of long-chain acyl-CoA esters in the liver samples was determined essentially according to the enzymatic method described by Kamiryo *et al.* (1979). The composition of the acyl chains in the liver acyl-CoA esters was determined as described by Prasad *et al.* (1987). Fifty mg of dry tissue was suspended in 0.5 ml of 100 mM sodium acetate (pH 4) with 10 mM  $\text{MgCl}_2$  plus 20  $\mu\text{l}$  of glacial acetic acid, 3.0 nmol of either 17:0-CoA or 20:0-CoA as internal standard, 100  $\mu\text{l}$  of 1% BHT in acetone, 1.5 ml of methanol and 0.7 ml of chloroform in a total volume of 2.8 ml. The tissue was homogenized at  $0.4^{\circ}\text{C}$  and further additions of chloroform and water were made to complete the extraction according to Bligh and Dyer (1959). The chloroform phase containing the major lipids was carefully removed and used for analysis of free fatty acids and phospholipids. In all subsequent steps 1% BHT in acetone was added to make a final concentration of 0.05%. The aqueous phase was washed twice with 1.5 ml portions of chloroform, and then 2 ml of acetonitrile was added to denature the protein. After standing for 20 min at room temperature the mixture was centrifuged and the supernatant was mixed thoroughly with 40 mg  $\text{Al}_2\text{O}_3$  for 15 min at  $20^{\circ}\text{C}$ . After removing the supernatant, the alumina was washed twice with 2 ml of chloroform:methanol (1:2) to remove adhering glycerolipids, and then with 1 ml acetone to facilitate drying.

After drying the alumina carefully under a stream of  $\text{N}_2$ , it was treated with 10–12 mg of sodium borohydride for 15 min, and then with 1 N HCl. Internal standards of 15:0 and 20:0 or 15:0 and 21:0 alcohols were added and the combined alcohols were extracted with pentane and dried under  $\text{N}_2$  in a tube and then treated with 5  $\mu\text{l}$  of *t*-butyldimethylsilyl (tBDMCS) reagent and 30  $\mu\text{l}$  of benzene for 20 min. Methanol (0.1 ml) was added to consume the excess reagent, and then 0.5 ml of water. The tBDMCS, ethers were extracted with pentane and, after evaporating the pentane, dissolved in 10  $\mu\text{l}$  of  $\text{CS}_2$ . For every analysis, solvent and reagent blanks were carried through the procedure to correct for impurities.

Samples were analyzed on a Helwett-Packard chromatograph equipped with a flame ionization detector. A  $180 \times 0.2$  cm glass column packed with 10% Silar 5CP (Supelco, Bellefonte, Pennsylvania) was operated with a  $\text{N}_2$  flow of 30 ml/min either isothermally at  $220^{\circ}\text{C}$  or temperature programmed from  $165$ – $220^{\circ}\text{C}$  at  $16^{\circ}\text{C}/\text{min}$ . The number of nmol of each acyl chain was calculated on the basis of its area relative to that for the added (21:0 or 20:0) alcohol internal standard. This value was then corrected for recovery of the C17:0-CoA standard that was added to all experiments. The method consistently gave with all added CoA esters, an overall absolute recovery of about 20% in the form of the alcohol silyl ether and no selective loss of



any acyl chain was observed. Thus the internal standards provide a valid index to the amount of each endogenous acyl CoA. The free fatty acids, phospholipids and triacylglycerols in the chloroform phase of the tissue extract were chromatographically separated by thin-layer chromatography on silicic acid using petroleum ether: diethyl ether: acetic acid (60:40:1). Each fraction was converted to methyl esters in the presence of added internal standards, and the acyl chain composition was determined by gas chromatography on a Silar 5CP column maintained at 220°C.

## Results

### *Sucrose-casein diets*

The non-esterified fatty acids in the livers of rats fed the fat-free sucrose-casein (SC) diet contained high amounts of 16:1, 18:1 and 20:3 (table 1) due to high induced levels of the  $\Delta^9$ -desaturase. In contrast, the relatively low amounts of 18:2 and 20:4 reflected the absence of the essential fatty acid, 18:2n-6, in the diet. Supplementation with corn oil caused large increases in the accumulated levels of 18:2 and 20:4 in cellular lipids and corresponding decreases in the endogenous acids derived from the action of the 9-desaturase, (16:1, 18:1 and 20:3). A similar compensatory decrease in these acids accompanied the addition of fish oil which increased the tissue levels of the n-3 acids, 20:5, 22:5 and 22:6.

The acyl-CoA esters contained major proportions of 18:0 (35–40%) and 20:4 (12–32%) with varied amounts of 18:1n-9, 18:2n-6, 20:3n-9 or 22:6n-3 depending on the diet supplement. The effects on the acyl-CoA by the diet alteration were quantitatively different from those for the non-esterified acids, and sometimes they were qualitatively different. For example, feeding corn oil gave a much greater increase in non-esterified linoleate (18:2) than arachidonate (20:4), while the increase in linoleoyl-CoA was much less than that for arachidonoyl-CoA. Similarly, when fish oil was added, the increase in non-esterified 20:5 exceeded that for 22:6 although the reverse occurred in the acyl-CoA pool. As a result, 20:5n-3 was poorly accumulated in the acyl-CoA fraction relative to its mol% in the diet or in the non-esterified acids.

The combined phospholipid fraction contained significant amounts of the  $\Delta^9$ -desaturase products, 18:1 (23%) and 20:3 (10%), and these were displaced when the n-6 or n-3 acids of corn oil or fish oil, respectively, were added to the diet. The triacylglycerols shifted similarly, but again in ways that reflected different selectivities among the various acids involved. They had a much more dramatic increase in 18:2n-6 than did the phospholipids, whereas the mol% of 20:4n-6 was only slightly increased in triacylglycerols. Although the added n-3 polyunsaturated acids of fish oil evoked a marked drop in the amount of the elongated n-9 acid, 20:3n-9, they did not appreciably displace the large amount of the shorter form (18:1n-9) from the

Table 1. Composition of acyl chains in liver lipids of rats fed diets based on SC.

Material analyzed		Fatty acid composition (mol%)										
Diet	Tissue abundance	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:4	22:5	22:6
Diets												
SC	—	—	—	—	—	—	—	—	—	—	—	—
SC+C	—	13.7	0	1.6	25.6	59.0	—	—	—	—	—	—
SC+F	—	20.0	12.6	3.4	13	1.4	1.1	2.0	14.1	—	1.1	8.2
Non-esterified fatty acids <sup>b</sup> (FFA)												
SC	1090±90	25±2	15±2	7.2±0.8	40±2	2.9±0.6	3.9±0.8	5±0.3	0	0.4±0.1	0	1.4±0.1
SC+C	3000±450	19±1	3±1	5.5±0.3	20±2	33±0.6	0.8±0.1	13±0.6	0	0.6±0.2	0.1±0.0	0.9±0.4
SC+F	1560±60	22±1	5±1	5.0±0.6	18±1	3.7±0.1	0.2±0.0	3±0.3	25±1	0.2±0.0	3.2±0.4	13.0±0.6
Acyl-CoA <sup>b</sup>												
SC	141±7	10.0±0.1	2.1±0.3	38±1	13.5±0.6	3.4±0.3	1.50±0.6	12±1	0	1.5±0.1	0.3±0.0	3±0.0
SC+C	141±7	10.4±0.5	0	35±1	5.2±0.4	12.8±0.9	0.9±0.4	32±2	0	0.8±0.2	0	2±0.2
SC+F	186±20	11.3±0.3	2.7±0.3	40±1	8.4±0.6	3.6±0.2	1.3±0.2	15±1	3.3±0.2	0.5±0.1	3.3±0.2	10.8±0.4
Phospholipids <sup>c</sup>												
SC	20	26	—	18	23	4.2	10.3	12	0.3	.1	1.5	4.5
SC+C	34	23	—	23	5.4	11.4	0.3	30	0.1	.7	1.5	4.7
SC+F	31	31	—	20	10.8	1.5	0.4	9	10.9	.2	2.7	14.0
Triacylglycerols <sup>c</sup>												
SC	11	37	—	1.2	41	2	4.9	0.1	0.1	0.5	1.0	0.1
SC+C	9	38	—	0.9	19	26	0.5	2.5	0	0.6	0.6	0.3
SC+F	5	32	—	0.9	30	6	0.7	0.5	9.2	0.8	4.8	12.6

<sup>a</sup>SC indicates the fat-free SC diet; + C indicates corn oil supplementation, and + F indicates fish oil supplementation.

<sup>b</sup>Tissue abundance expressed as nanomoles per g wet weight of liver; mean ± SEM of 4 determinations.

<sup>c</sup>Tissue abundance expressed as micromoles per g wet weight of liver; mean of two independent determinations.

weight) than we had observed with the SC diets (1000-5000 nmol per g) and somewhat higher amounts of acyl-CoA esters (230 vs 135 nmol per g). The composition of the various lipid pools differed in ways that could be related to the presence of 32 mol% 18:2 in the lipids of the chow diet. We noted that fasting the chow-fed animals overnight raised the abundance of free fatty acids in liver to about 6000 nmol per gram while diminishing the risk that they were derived directly from the dietary fat. Because we were testing for selectivities in acyl chain turnover in the liver, we elected to study the chow-fed animals with an overnight period of fasting prior to obtaining samples for analysis.

The non-esterified fatty acids in the livers of the fasted rats contained more oleate (18:1) and arachidonate (20:4) and much more linoleate (18:2n-6) than was in the non-fasted animals (table 2) while exhibiting a compensatory drop in the mol per cent of palmitate (16:0). While fasting caused the mol% of 18:2 to increase also in the acyl-CoA esters, the 18:2 decreased in the phospholipids and triacylglycerols as the mol% of 20:4 rose relative to values from the non-fasted rats. When the effects of oil-supplementation were compared among the non-esterified acids after an overnight fast, the corn oil produced no appreciable change whereas the fish oil led to decreased 18:2 and 20:4 as these acids were displaced by the dietary n-3 acids, 20:5, 22:5 and 22:6.

Upon supplementing with corn oil, the acyl-CoA esters had an elevated mol% of 18:2 while 18:0 and 20:4 decreased slightly. Similarly, fish oil supplementation elevated the mol% of the dietary polyunsaturated acids, 20:5n-3 and 22:6n-3, in CoA esters while decreasing the mol% of 20:4. The phospholipids and triacylglycerols exhibited changes that qualitatively mirrored those noted for the non-esterified acids and the CoA esters. The n-3 dietary acids (or their related derivatives) tended to increase in these glycerolipids with a compensatory decrease in the amount of the (n-6) type.

## Discussion

To facilitate comparisons of the relative selectivities that occurred during the changes in acyl chain composition, the results are also presented in table 3 in the form of ratios of the mol% of an acid in a product pool compared to that in the possible precursor pool. For example, the ratio for a non-esterified fatty acid to its abundance in the diet indicates in part the extent to which the acid may tend to be derived from the tissues of the animal rather than the diet. Ratios less than 1 for 18:2 during corn oil supplementation are expected because the diet is very rich in this acid, but the value of 2.6 for 18:2 during fish oil supplementation of the fat-free diet (table 3) suggested that there was a fairly vigorous displacement or mobilization of tissue reservoirs of 18:2n-6 when it was not very abundant in the diet and other polyunsaturated acids were. A similar mobilization of arachidonate, 20:4n-6, by the fish oil supplementation may reflect competition for esterification by the 20:5n-3 (with phospholipid/acyl-CoA ratios of 2.8-3.3). A similar displacement by n-3 acids was also observed in dog plasma lipids during fish oil supplementation which increased the amount of circulating 18:2n-6 and 20:4n-6 during the first week of shifting to a diet rich in n-3 acids (B. Culp and W. Lands, unpublished results; Culp *et al.*, 1980). The triacylglycerols in plasma retained an elevated mol% of 20:4n-6

Table 2. Composition of acyl chains in liver lipids of rats fed chow-based diets.

Tissue abundance <sup>b</sup>	Fatty acid composition (mol%)										
	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:4	22:5	22:6
<i>Saturated fatty acids (FFA)</i>											
240±30	39±2	10±1	5.5±0.2	15±2	12±1	0.9±0.1	7.5±0.7	2.9±0.2	0	2.3±0.2	4.0±0.6
6100±1000	20±1	2.0±0.1	7.0±0.9	23±1	30±2	0.6±0.1	14.5±0.1	0.8±0.1	0.6±0.1	0.5±0.2	3.3±0.7
5700±1000	22±2	0.9±0.1	7.9±1	20±1	31±3	1.0±0.1	14±	0.9±0.0	2.0±0.3	0.1±0.1	1.±0.1
6300±100	22±1	4.0±0.5	4.7±0.5	18±1	18±1	0.2±0.0	5.5±0.7	7.2±0.4	0.3±0.1	3.6±0.1	17±1
<i>Unsaturated fatty acids</i>											
230±16	10.8±0.4	0	3.8±0.5	5.2±0.1	1.5±0.5	2.0±0.2	2.3±1	0.4±0.1	2.6±0.1	2.0±0.2	4.2±0.1
150±30	5.8±0.2	0.5±0.1	3.7±3	5.5±1	2.6±4	0.8±0.1	2.0±3	—	0.9±0.1	0.9±0.2	1.3±0.4
130±13	7.1±0.4	0.2±0.1	3.1±1	6.0±0.4	3.6±2	0.9±0.1	1.7±1	—	0.4±0.0	0.5±0.3	1.0±0.1
130±25	9.0±0.4	1.1±0.1	3.5±2	7.3±2	2.5±2	1.5±0.1	1.2±1	1.1±0.4	0.9±0.3	0.9±0.2	4.3±0.6
<i>Isoprenoids</i>											
30	2.5	—	2.2	5.0	14.5	0.8	2.0	1.4	0.6	1.5	7.0
66	1.9	—	2.9	6.3	8.6	0.8	2.9	0.2	0.4	0.7	5.8
60	1.8	—	2.7	6.5	12.2	0.3	2.8	0.1	0.5	1.1	6.1
54	2.5	—	2.5	6.0	8.5	0.5	1.5	3.1	0.1	1.7	15.0
<i>Phospholipids</i>											
2.4	4.2	—	2.1	3.2	16.6	0.3	1.9	0.7	0.6	0.8	1.4
2.2	3.8	—	8.9	3.6	3.4	0.3	7.9	—	0.6	0.5	1.5
7.0	2.7	—	5.4	26.4	32.0	0.4	4.8	0.2	1.0	0.9	0.9
4.6	3.3	—	4.7	28.5	11.1	0.2	2.6	3.4	0.7	3.2	10.3

are consumed *ad libitum* with an 18 h fast prior to killing the rats for analysis, with the exception of those designated Chow-NF which were not fasted. al fats are indicated as C for corn oil and F for fish oil.

ances are in the same terms as in table 1.

**Table 3.** Selectivity ratios indicating the relative enrichment of an acyl chain between two lipid pools.

Ratio <sup>a</sup>	Diet fed	16:0 C <sup>b</sup>	18:0 C F	18:1 C F	18:2 C F	20:4 C F	20:5 C F	22:6 C F
FFA Diet	Chow-NF <sup>c</sup>	1.8	0.8	0.5	0.4	37	14.5	13.
	Chow	1.0	1.0	0.8	0.9	72	4.	11.
	Chow + Suppl	1.3 1.0	1.1 1.0	0.7 0.9	0.6 1.3	3.7	—	0.9 10 2.9
	SC <sup>d</sup> + Suppl	0.5 1.1	3.4 1.5	0.8 2.6	0.5 2.6	1.5	—	1.8 — 1.6
Acyl-CoA FFA	SC	0.4	5.2	0.3	1.2	2.4	—	2.1
	Chow-NF	0.3	6.9	0.3	1.3	3.0	0.13	1.0
	Chow	0.3	5.3	0.3	0.9	1.4	—	0.4
	Chow + Suppl	0.3 0.4	3.9 7.4	0.3 0.4	1.1 1.4	1.3 2.1	—	0.15 1.0 0.25
Phospholipid Acyl-CoA	SC + Suppl	0.5 0.5	6.4 7.9	0.3 0.5	0.4 1.0	2.5 4.7	—	0.13 1.8 0.8
	SC	2.6	0.5	1.7	1.2	1.0	—	1.5
	Chow-NF	2.3	0.6	1.0	1.0	0.9	3.5	1.7
	Chow	3.3	0.8	1.1	0.3	1.4	—	4.5
	Chow + Suppl	2.6 2.7	0.9 0.7	1.1 0.8	0.3 0.3	1.6 1.3	—	2.8 6.1 3.5
	SC + Suppl	2.2 2.7	0.7 0.5	1.0 1.3	0.9 —	0.9 0.6	—	3.3 2.4 1.3

<sup>a</sup>The ratios indicated were derived from the mol% values given in tables 1 and 2.

<sup>b</sup>C indicates corn oil supplementation, and F indicates fish oil supplementation.

<sup>c</sup>Chow-NF indicates the non-fasted animals receiving unsupplemented chow diets as noted in table 2.

<sup>d</sup>SC indicates the sucrose-casein diet noted in table 1.

ferred a preference by the 1-GPC: acyl-CoA-GPC: acyltransferase of rat liver for transfer of the 20:5n-3. The present results confirm that selectivity, with ratios for phospholipid/acyl-CoA that were consistently higher for 20:5n-3 (3.5, 2.8, 3.3) than 20:4n-6 (0.9, 1.2, 0.6).

High selectivity ratios for acyl-CoA/non-esterified acid may indicate that an acyl group enters the acyl-CoA pool with more facility or speed than it leaves. The particularly high value for 18:0 (4-8) contrasts sharply with the low values for 16:0 and 18:1 (0.3-0.5). Induction of hepatic fatty acid synthetase (up to 50-fold) by dietary variations (Flick *et al.*, 1977; Bernert and Sprecher, 1977) was much greater than that for microsomal elongation of 16:0-CoA to 18:0-CoA (2.5-fold). Since the synthetase produces mainly palmitate (Burton *et al.*, 1968), a higher mol% of 16:0 compared to 18:0 might be expected. The very high mol% of 18:0 in the acyl-CoA fraction strongly suggests that this acid enters the acyl-CoA pool more readily than it leaves. The microsomal acyl-CoA hydrolase activity is apparently two times higher for 16:0-CoA than 18:0 CoA (Jezyk and Hughes, 1971), but no difference between these was obtained for phosphatidate synthesis (Possmayer *et al.*, 1969). A further check at the exit of these two acids from the acyl-CoA pool seems desirable. It will be especially important to examine whether the relatively higher content of 18:0 CoA and 20:4 CoA influences the formation of selected species of phosphatidyl inositol. The low ratios of acyl-CoA to non-esterified acid for 20:5 suggest that this acid tends to leave the thiol ester pool more readily than it enters. Put in another way, the 20:5n-3 appears to accumulate more in the non-esterified form than as the acyl-CoA, whereas the 20:4n-6 appears to accumulate more as acyl-CoA than as the non-esterified form. These combined tendencies could have an interesting impact upon the availability of the eicosanoid-forming oxygenases to convert the nonesterified 20:4n-6 to biologically active materials (Bergstrom *et al.*, 1964; Murphy *et al.*, 1979). Those oxygenases act on the non-esterified acids, and 20:5n-3 can antagonize that process (Lands *et al.*, 1973; Lands and Byrnes, 1982).

The relatively low ratio for acyl-CoA/nonesterified acid observed for 18:2 when a sucrose-casein diet was supplemented with corn oil (0.4) needs to be interpreted in terms of competition for the limited CoASH thiol group. The competition by 13-15 mol% non-esterified 18:2 (tables 1 and 2) with 13-15 mol% non-esterified 20:4 to a greater mol% of 20:4-CoA with the sucrose-casein diet, but to relatively low mol% of 18:2-CoA with the chow diet. Apparently other regulatory features occur during the alteration in diet, which might include altered cellular amounts of the acyl-CoA:cholesterol-specific ligase (Laposta *et al.*, 1985) or different amounts of lipid receptors that permit facile transfer of linoleate from the acyl-CoA pool (*e.g.* transfer of linoleate to diacylglycerol to form triacylglycerol). The decreases in the mol% of 18:1 and 20:3 when the diets were supplemented with added polyunsaturated fat is consistent with a decreased induction (Prasad and Joshi, 1979) or inhibition (Jeffcoat and Lands, 1978) of the 9-desaturase as well as with competitive displacement of the 18:1 by its products from the finite cellular lipid pools by the polyunsaturated acids. The greater effect on the elongated form, 20:3, suggests that a particularly effective competition in the acyl-CoA pool by the polyunsaturated acids may prevent the conversion of 18:1-CoA to 20:3-CoA.

The relatively high selectivity ratios for phospholipid/acyl-CoA for 22:6n-3 with fasted animals (3.5, 4.5, 6.1) suggest that this acid may move more readily from the

1:3, 2:4). Again the results suggest the possibility of additional factors controlling the selective action of the acyltransferases during fasting. Such an action could result in vigorous salvage of this highly unsaturated fatty acid during times of limited supply, and it may be involved in the apparent ability of tissues to retain 22:6n-3 more effectively than any other polyunsaturated acid. The selectivity ratios of 10, 11 and 13 for 22:6 in non-esterified acids relative to the diet indicate very active mobilization of the endogenous acid. If this fatty acid does indeed have an important role in membrane function (Neuringer *et al.*, 1984) its selective retention would clearly have survival value.

Many years ago we approached the question of selective acyl transfer by measuring the abundances of the molecular species of phosphatidylcholine in rat liver during fasting followed by feeding a fat-free (SC) diet (Lands and Hart, 1966). The results in that study are confirmed here in that during fasting the chow-fed rats accumulated higher mol% of the tetraene 04\* and hexaene 06 molecular species of lecithin in the liver. Then the subsequent fat-free diet induced high levels of fatty acid synthetase and  $\Delta 9$ -desaturase to produce large amounts of 16:1n-7 and 18:1n-9 and their corresponding 01 and 11 molecular species with a relative loss from the liver of the 02, 04 and 06 molecular species in the absence of dietary polyunsaturated acids. The dietary shift apparently led to the export of many polyunsaturated acids from the liver and their replacement by monoenoic acids in a way that created an apparent local deficiency of essential fatty acid. That selective export apparently does not have this consequence during fasting when the monoenoic acids are not as abundant.

The use of the sensitive subnanomole assay for determining the acyl chains in the acyl-CoA pool has allowed us to see shifts in composition due to diet changes. Our results have shown that it is possible to discern selective movements of acyl chains into and out of the acyl-CoA pool of liver. The application of the method to a primary culture of hepatocytes could permit frequent sampling at closely spaced time intervals so that more precisely defined selective flux rates could be determined. We expect to see continued study of this very small pool of dynamically metabolized thiolesters as biochemists examine the important contributions of polyunsaturated fatty acids to human health.

## Acknowledgements

This research was supported in part by grants from the United States Public Health Service (AM05310 and HL-34045) and a fellowship (B. C.) from the International Association of Fish Meal Manufacturers. The principal investigator (W. E. M. L.) received a Pfizer Biomedical Research Award.

## References

- Bergstrom, S., Danielsson, H. and Samuelsson, B. (1964) *Biochim. Biophys. Acta*, **90**, 207.
- Bernert, J. T. Jr. and Sprecher, H. (1977) *J. Biol. Chem.*, **252**, 6736.

\*The designations 04, 06, 01, 11, etc. indicate molecular species of lecithin for which the number of double bonds in fatty acids at positions 1 and 2 are noted (Lands and Hart, 1966).

- Burton, D., Haavik, A. G. and Porter, J. W. (1968) *Arch. Biochem. Biophys.*, **126**, 141.
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem.* **37**, 911.
- Culp, B. R., Lands, W. E. M., Lucchesi, B. R., Pitt, B. and Romson, J. (1980) *Prostaglandins*, **20**, 1021.
- Ellingson, J. S., Hill, E. E. and Lands, W. E. M. (1970) *Biochem. Biophys. Acta*, **196**, 176.
- Flick, P. K., Chen, J. and Vagelos, P. R. (1977) *J. Biol. Chem.*, **252**, 4242.
- Groot, P. H. E., Scholfe, H. R. and Hulsmann, W. H. (1976) *Adv. Lipid Resh.*, **14**, 75.
- Hill, E. E. and Lands, W. E. M. (1970) in '*Lipid metabolism*' (ed. S. J. Wakil) (New York: Academic Press Inc.) p. 185.
- Holub, B. J. (1976) *Lipids*, **11**, 1.
- Jeffcoat, R. and James, A. T. (1978) *FEBS Lett.*, **85**, 116.
- Jczyk, P. F. and Hughes, H. N. (1971) *Lipids*, **6**, 107.
- Kamiryo, T., Nishikawa, Y., Mishina, M., Terao, M. and Numa, S. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4390.
- Lands, W. E. M. (1979) in '*Geometrical and positional fatty acid isomers*' (eds E. A. Emken and H. J. Dutton) (Champaign: American Oil Chemists Society) p. 181.
- Lands, W. E. M. and Byrnes, M. J. (1982) *Prog. Lipid Res.*, **20**, 287.
- Lands, W. E. M. and Crawford, C. G. (1975) in '*Membrane bound enzymes*' (ed. A. Martonosi) (New York: Plenum Press Inc.) p. 3.
- Lands, W. E. M. and Hart, P. (1966) *J. Am. Oil Chem. Soc.*, **43**, 290.
- Lands, W. E. M., Inoue, M., Sugiura, Y. and Okuyama, H. (1982) *J. Biol. Chem.*, **257**, 14968.
- Lands, W. E. M., LeTellier, P. E., Rome, L. H. and Vanderhoek, J. Y. (1973) *Adv. Biosci.*, **9**, 15.
- Laposta, M., Reich, E. L. and Majerus, P. W. (1985) *J. Biol. Chem.*, **260**, 11016.
- Marcel, Y. L. and Suzue, G. (1972) *J. Biol. Chem.*, **247**, 4433.
- Murphy, R. C., Hammarstrom, S. and Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4275.
- Neufeld, E. J., Wilson, D. B., Sprecher, H. and Majerus, P. W. (1983) *J. Clin. Invest.*, **72**, 214.
- Neuringer, M., Connor, W. E., Van Pellen, C. and Barstad, L. (1984) *J. Clin. Invest.*, **73**, 272.
- Okuyama, H., Lands, W. E. M., Christie, W. W. and Gunstone, F. D. (1969) *J. Biol. Chem.*, **244**, 6514.
- Possmayer, F., Scherphof, G. L., Dubbelman, T. M. A. R., Van Golde, L. M. G. and Van Deenen, L. L. M. (1969) *Biochem. Biophys. Acta*, **176**, 95.
- Prasad, M. R. and Joshi, V. C. (1979) *J. Biol. Chem.*, **254**, 997.
- Prasad, M. R., Sauter, J. and Lands, W. E. M. (1987) *Anal. Biochem.*, (in press).
- Yamada, K. and H. Okuyama (1978) *Arch. Biochem. Biophys.*, **190**, 409.





# Importance of determining viability of *Mycobacterium leprae* inside macrophages—an *in vitro* method using uracil

SUNANDA VEJARE and P. R. MAHADEVAN

The Foundation for Medical Research, 84-A, R. G. Thadani Marg, Worli, Bombay 400 018, India

**Abstract.** It has been demonstrated that *Mycobacterium leprae*, are capable of taking up uracil and incorporating it into trichloroacetic acid-insoluble materials, both as free suspension of bacteria, as well as when they are inside the macrophages, a host cell for their *in vivo* survival. Same amount of bacteria show better incorporation inside macrophages than as free bacterial suspension. Both types of incorporation are inhibited by rifampicin an antileprosy drug and an RNA synthesis inhibitor. Thus uracil uptake by *Mycobacterium leprae* inside macrophages has been used for standardising a rapid *in vitro* viability assay for the leprosy causing bacteria.

**Keywords.** Uracil uptake; *Mycobacterium leprae*; macrophages; viability assay.

## Introduction

*Mycobacterium leprae*, the causative organism for leprosy is an intracellular obligate pathogen. In patients suffering from lepromatous leprosy the bacteria are seen inside several host cells, but primarily macrophages in various tissues and schwann cells of peripheral nerves. These two cell types are important for immune functions and normal nerve functions, respectively. It is well documented that those susceptible to lepromatous leprosy, have poor cell mediated immunity (Bloom and Mehra, 1984) a function in which macrophages play an important role as antigen presenting cell. The patients also suffer from failure of nerve function leading to neuropathy, basically expressed as features related to damage in the myelin of myelinated nerves and the schwann cells of unmyelinated peripheral nerves (Shetty *et al.*, 1980). Thus the understanding of host-pathogen interaction is basic necessity in explaining the pathogenesis in leprosy especially lepromatous leprosy.

The information available regarding metabolism of *M. leprae* was fully described recently by Wheeler (1984). The nucleic acid metabolism as studied by the uptake and incorporation of labelled pyrimidines and purines was also described by him. In analysing the reported ability of *M. leprae* from armadillo to incorporate the precursor of nucleic acid (Khanolkar *et al.*, 1978; Drutz and Cline, 1972; Prasad and Nath, 1981; Nath *et al.*, 1982; Khanolkar and Wheeler, 1983) it was concluded by Wheeler (1984) that purines like, hypoxanthine and adenosine were better precursors than pyrimidines like thymidine or uracil. However it is noteworthy that all experimental evidence by Nath and her colleagues, cited above, clearly indicate utility of thymidine as a metabolic precursor for determining viability and drug sensitivity of *M. leprae*, phagocytosed by macrophages. There are other *in vitro* assay systems for viability of *M. leprae* have also been described. They include the

dihydroxyphenylalanine (DOPA) uptake (Ambrose *et al.*, 1978) and ATP assay system (Dhople and Hanks, 1980).

Viability of *M. leprae* inside the host cells cannot be judged by size, shape, morphology or staining property of the bacteria. Such a procedure has always been arbitrary. Thus the dynamic metabolism expressed by *M. leprae* inside the host cell has to be used. Once such assay systems are well established, we could use them for determining the nature of *M. leprae* under various conditions that affect the viability. Such conditions involve exposure to antileprosy drugs, exposure to immune competent cells (lymphocytes) and cell products (lymphokines). These products could alter the viability of *M. leprae*. Thus it is essential to have such assay systems and some of them have already been identified (Nath *et al.*, 1982; Ambrose *et al.*, 1978; Vithala *et al.*, 1983; Mankar *et al.*, 1984).

In this paper we describe use of uracil as a precursor to assess the metabolism of *M. leprae*, inside macrophages. Potential use of uracil for determining viability of mycobacteria like BCG, was identified by Rook *et al.* (1981), who later adopted it to show killing of BCG inside macrophages by activated lymphocytes (Rook *et al.*, 1985). We have adopted a closely similar procedure with *M. leprae*.

This paper also presents detailed data to demonstrate that *M. leprae* are able to incorporate uracil even as free suspension and the two systems one as the free suspension and other inside host cells have been compared.

## Materials and methods

### *Preparation of N-2 Hydroxypiperarine N-2 ethane sulphonic acid Buffer*

N-2 Hydroxypiperarine N-2 ethane sulphonic acid (HEPES, Sigma Chemicals Co., USA) Powder—0.15 M, NaCl—0.15 M and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.1 mM were dissolved to make a 100 ml solution with distilled water; and pH was adjusted to 7.2 with 1N HCl, autoclaved (15 lbs./121°C/30 min) and stored for use.

### *Preparation of M. leprae*

*M. leprae* was obtained from tissue of leprosy patients as per the method of Ambrose *et al.* (1978). Bacteria obtained were acid fast stainable and were used only after ascertaining that they were free from other rapidly growing contaminating bacteria, including other mycobacterial strains. *M. leprae* normally does not grow in the common mycobacterial medium.

### *Assay of [ $^3\text{H}$ ]-uracil incorporation in M. leprae*

[ $^3\text{H}$ ]-Uracil labelled in positions 5–6 was obtained from Amersham Radiochemical Centre, London, (sp. act. 35 Ci/mmol). Reaction mixture containing  $20 \times 10^6$  bacteria, HEPES Buffer, pH 7.2 and 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]-uracil in a final volume of 0.5 ml/

bes were centrifuged at 5000 *g*. To the residue, 20% cold trichloroacetic acid (TCA) was added. The precipitated material obtained after acid addition, was filtered through Millipore filter (0.22  $\mu$ m pore size) which was then washed with methanol and dried. Use of methanol was just to remove the aqueous layer on the filter only. The filter paper was placed in vial containing 10 ml of Bray's scintillation fluid (Bray, 1960), and with the help of Kontron MR 300 scintillation counter the radioactivity (dpm) of [ $^3$ H]-uracil in TCA insoluble material was reported.

#### Effect of drugs on incorporation

While the bacteria were being incubated with the labelled precursor [ $^3$ H]-uracil; they were exposed to Dapsone (DDS) or rifampicin at concentration ranging from 0.5  $\mu$ g–10  $\mu$ g/ml. This was to find out whether the known anti-*M. leprae* drugs can block the metabolism of *M. leprae* and thus the incorporation of the precursor. DDS was obtained from Burroughs Wellcome, Bombay and rifampicin from Sigma Chemical Co., St. Louis, Missouri, USA.

#### Macrophage preparation

Macrophages were obtained from Swiss white mice. The mice were injected with 5 ml minimum essential medium (MEM) containing inactivated human AB type serum (20% level) into peritoneal cavity, after killing the animal by cervical dislocation. The peritoneal fluid was collected from the animal by agitating the cavity with the medium and 0.7 ml of the collected fluid containing the cells was distributed in Leighton tubes. Tubes were then incubated at 37°C in 5% CO<sub>2</sub> atmosphere. The medium was changed every 24 h, so as to remove non adherent cells and the cell culture was thus matured for 3 days. This resulted in fairly good monolayers of adherent macrophages which were phagocytic and largely free from other types of cells.

#### [ $^3$ H]-Uracil incorporation studies with macrophages containing *M. leprae*

After getting the monolayer of adherent macrophages on the flat side of Leighton tubes (3 day old cultures), the tubes were divided in 3 sets. One set was infected with viable *M. leprae* and another with heat killed *M. leprae* ( $5 \times 10^6$  from human or marmoset tissue). The third set did not receive any *M. leprae* and was used as a control. After 24 h, all tubes were labelled with 1  $\mu$ Ci of [ $^3$ H]-uracil. After 6 days of incubation at 37°C in 5% CO<sub>2</sub> atmosphere, the cultures were terminated. Medium was drained from all tubes and one ml of fresh saline was added and tubes were kept cold (4°C) for 15 min. The cells were scrapped from the surface of glass with

In experiments similar to those described above, cultures exposed to *M. leprae* ( $5 \times 10^6$ /tube heat killed and live) and the precursor ( $[^3\text{H}]$ -uracil) after incubation for 6 days were treated differently. The macrophages after recovery were lysed by freezing and thawing (8 cycles). The tissue debris was separated from bacteria by centrifugation at 1000 *g* for 15 min followed by bacterial separation by centrifuging at 5000 *g* for 30 min. Bacterial count was determined microscopically with acid fast staining by the Ziehl Neelsen method (Cruickshank, 1968). After treating the bacteria with 20% cold TCA, the incorporation associated with the precipitated bacteria was recorded as dpm/ $1 \times 10^6$  *M. leprae*.

#### *Inhibition of incorporation by rifampicin*

The *M. leprae* that were used to infect the macrophages were preincubated for 3 days with rifampicin (10  $\mu\text{g/ml}$ ) and added to macrophage cultures which were earlier exposed to rifampicin (10  $\mu\text{g/ml}$ ) for 24 h before the addition of *M. leprae*. The drug concentration was kept as 10  $\mu\text{g/ml}$  since we wanted a definite action on the bacteria and the minimum inhibitory concentration with this type of assay system is yet to be worked out. The cultures were then exposed to precursor  $[^3\text{H}]$ -uracil and incubated in the presence of rifampicin (10  $\mu\text{g/ml}$ ). The control cultures had no drug treatment either to the bacteria or macrophages. The incorporation of the precursor was determined in the bacteria as described earlier.

### **Results**

Six different *M. leprae* isolates from human leprosy nodules and two different *M. leprae* preparations from infected armadillo tissue showed incorporation of  $[^3\text{H}]$ -uracil in TCA insoluble product, when  $20 \times 10^6$  bacteria were incubated with 1  $\mu\text{Ci}$  of labelled uracil. In all the cultures, the live *M. leprae* showed significantly higher incorporation than formalin treated *M. leprae* (table 1). However the formalin treated bacteria did not show low level of incorporation, consistently in all experiments, even though the level in each experiment was much less than that of the corresponding sample with live *M. leprae*.

In these experiments  $20 \times 10^6$  bacteria were used. Before this was adopted, the level of incorporation when different number of bacteria exposed to same amount of uracil was also determined and this showed that increasing bacterial level resulted in higher incorporation, though not at a linear rate (data not presented). The non linearity may be due to different level of viability in each suspension since homogenous distribution of clumpy *M. leprae* is difficult.

#### *Inhibition of uptake by drugs*

The uracil uptake by the bacteria was indicated as an apparent metabolic function by the inhibition of uptake by streptomycin, M. leprae, and PDS. This inhibition was

**Table 1.** Incorporation of [<sup>3</sup>H]-uracil (as dpm) by *M. leprae* (armadillo and human).

Patient's name (Initials)	Formaldehyde killed <i>M. leprae</i> (F)	Live <i>M. leprae</i> (L)	Net uptake (L-F)	BI/MI
M.P.	466	3469	2997	6+, 3%
L.G.	181	6582	6401	6+, 1%
M.B.	1454	5463	4009	1+, 1%
M.P.	2051	4539	2488	6+, 1%
A.B.	2147	3627	1480	4+, -
M.G.	103	3786	3683	6+, 4%
Ar-1	661	4570	3909	6+, 10%
Ar-2	3804	6439	2635	6+, 35%
Mean ± S.D.	1358 ± 1278	5241 ± 549	3431 ± 1368	

All values are average of duplicates.

Radioactivity is expressed in dpm/20 × 10<sup>6</sup> *M. leprae*.

BI, Bacterial Index (Bacillary load).

MI, Morphological Index (live).

Difference between killed and live *M. leprae* is significant. *P* < 0.001.

S.D., Standard deviation.

**Table 2.** Incorporation of [<sup>3</sup>H]-uracil by *M. leprae* (Armadillo) represented as dpm/20 × 10<sup>6</sup> *M. leprae* in the presence of antileprosy drugs (DDS and rifampicin).

	Ar-1 (Nodule)	Ar-2 (leproma)	Ar-3 (Spleen)*	Mean ± S.D.
Formaldehyde (8%) killed	1321	857	176	784 ± 575
Live <i>M. leprae</i>	8150	7215	1641	5668 ± 3507
Live <i>M. leprae</i> + DDS (0.1 µg/ml)	1066	914	447	809 ± 322
DDS (1 µg/ml)	1216	538	301	685 ± 474
DDS (10 µg/ml)	1843	536	219	866 ± 857
Rifampicin (0.5 µg/ml)	1268	434	241	647 ± 545
Rifampicin (2 µg/ml)	948	538	176	554 ± 386
Rifampicin (10 µg/ml)	780	437	233	483 ± 276

\*The data with armadillo *M. leprae* from spleen (Ar-3 spleen) is an average value of 3 separate experiments from 3 separate spleen tissues.

All values are average of duplicates (in all other experiments) 20 × 10<sup>6</sup> *M. leprae* was used.  
S.D., Standard deviation.

g armadillo derived *M. leprae*, the inhibitory activity on incorporation was nonstrable by 0.1–10 µg/ml of DDS and 0.5–10 µg/ml rifampicin added for the ubation mixture. The inhibition was revealed by lowered level of precursor orporation in the drug treated *M. leprae* as compared to the live control *leprae*.

*leprae* from clinically relapsed and normal patients

showed no susceptibility to DDS and rifampicin. This was indicated by the level of incorporation by *M. leprae* in the presence of these drugs remaining similar to that of live *M. leprae* (table 3). On the other hand *M. leprae* from armadillo and some untreated patients were susceptible to DDS and rifampicin.

**Table 3.** Incorporation of [ $^3\text{H}$ ]-uracil by *M. leprae* (human) in presence of antileprosy drugs (DDS and rifampicin).

BI/MI clinical assessment	FMR 738 6+, 1% relapsed	FMR 1431 54+, 4% untreated	FMR 722 6+, 1% 1y. treated	Mean $\pm$ S.D.
Formaldehyde (8%) killed	200	2241	382	940 $\pm$ 1128
Live <i>M. leprae</i>	4534	4173	12002	6902 $\pm$ 4418
Live <i>M. leprae</i> + DDS (0.1 $\mu\text{g/ml}$ )	2732	3554	9913	5332 $\pm$ 3977
DDS (1 $\mu\text{g/ml}$ )	3323	3971	10135	5808 $\pm$ 3758
DDS (10 $\mu\text{g/ml}$ )	3639	3515	11846	6332 $\pm$ 4773
Rifampicin (0.5 $\mu\text{g/ml}$ )	2570	3459	12144	6056 $\pm$ 5288
Rifampicin (2 $\mu\text{g/ml}$ )	3000	4649	11579	6408 $\pm$ 5432
Rifampicin (10 $\mu\text{g/ml}$ )	2990	4460	16700	8049 $\pm$ 7526

All values are average of duplicates.

Quantity of uracil incorporates is expressed as dpm/20  $\times 10^6$  *M. leprae*.

S.D., Standard deviation.

BI/MI, Bacterial Index (load)/Morphological Index (live).

### *Uptake of uracil by M. leprae phagocytosed by macrophages*

It was interesting to determine the level of uracil taken up by *M. leprae* phagocytosed by the macrophages, so as to compare with the ability of free *M. leprae*. The experiment with armadillo *M. leprae* phagocytosed by macrophages showing the level of incorporation in  $1 \times 10^6$  macrophages indicated, that discernable level of incorporation was consistent in all the 8 experiments, even though the level of incorporation was different in each of the experiment. The level of *M. leprae* used was same in all cases ( $5 \times 10^6$  bacteria) and equal amount of phagocytosis by the macrophages is assumed to occur (table 4).

### *Increase in uptake of precursor by M. leprae inside the macrophages*

Earlier, different concentration of *M. leprae* was used in free suspension with the precursors and we found, that the incorporation was insignificant and not discernable when the *M. leprae* used was less than  $5 \times 10^6$  (data not presented). Thus it was necessary to compare the level of incorporation of uracil by *M. leprae* in a quantum less than  $5 \times 10^6$  as free bacteria in suspension and same amount of *M. leprae* phagocytosed by macrophage cultures. Data provided (table 5) show level of incorporation by  $1 \times 10^6$  *M. leprae* under the above two different conditions is significantly different. *M. leprae* ( $1 \times 10^6$ ) inside the macrophages showed several fold higher incorporation indicating a facilitation of increased uptake of uracil by *M. leprae* when they were inside the macrophages.

**Table 4.** Experimental data showing incorporation of [ $^3\text{H}$ ]-uracil (as dpm) by *M. leprae* inside macrophages.

Experiment No. (armadillo)	Macrophage only	Macrophage + heat killed <i>M. leprae</i> (B)	Macrophage + live <i>M. leprae</i> (C)	(C-B)
1	80	420	1600	1180
2	80	160	700	540
3	585	379	711	332
4	148	294	451	157
5	—	331	445	114
6	—	1384	2382	998
7	—	1330	2480	1150
8	—	2660	3080	420

$P < 0.01$

Radioactivity is expressed as dpm/ $1 \times 10^6$  macrophages. *M. leprae*  $5 \times 10^6$ /tube.

**Table 5.** Comparison of incorporation by *M. leprae* (armadillo or human) in buffer and inside macrophages as dpm/ $1 \times 10^6$  *M. leprae*.

Source of <i>M. leprae</i>	Incorporation in buffer by $1 \times 10^6$ <i>M. leprae</i> (L-F) (A)	Incorporation inside macro- phage by $1 \times 10^6$ <i>M. leprae</i> (L-HK) (B)	Difference (inside Mφ) (in buffer) (B - A)
Ar (splcen)	174	511	337
Ar (splcen)	0	538	538
Ar (splcen)	20	1685	1665
Ar (leproma)	7	786	779
Human	0	87	87

$P < 0.05$

L-F Live, formalin treated.

L-H.K.—Live, heat killed *M. leprae*.

### *Inhibition of such increased uptake by drugs*

The increased incorporation of uracil by *M. leprae* while they were inside the macrophages was tested for inhibition with rifampicin. Data in table 6 show that in the presence of rifampicin (10  $\mu\text{g}/\text{ml}$ ) the increased incorporation of uracil is drastically reduced indicating that the uptake is a true metabolic process.

## Discussion

It is very likely that uracil is not a preferred metabolite of *M. leprae* towards nucleic acid metabolism as suggested by Wheeler *et al.* (1984). However as compared to low incorporation obtained in armadillo derived bacteria by Khanolkar and Wheeler



Live <i>M. leprae</i> only	2950	1705	1140	994	significant $P < 0.025$ significant
+ Rifampicin (10 µg/ml) treated <i>M. leprae</i>	659	577	320	272	

\*From patients.

Incorporation is expressed as dpm/ $1 \times 10^6$  *M. leprae*.

incorporation of radioactivity significant enough to measure as acid insoluble component. However as a quantity in p mol, the incorporation is very low. Several observations show that the uptake of uracil is a metabolic activity. The uptake is extremely low in formalin treated bacteria. The uptake is proportional to bacteria exposed to the metabolite and also dependent on period of incubation, and further it is blocked by anti *M. leprae* drugs like DDS and more particularly by rifampicin, an inhibitor of RNA synthesis. The incorporation of uracil was first demonstrated by *in vitro* grown acid fast bacteria derived from leprosy nodule (data not presented) and later tested with *M. leprae* from leprosy nodules as well as infected armadillo tissues. But results presented here are only with *M. leprae*, from the human and armadillo tissues because of obvious interest in this group of pathogenic mycobacteria.

The clinically relapsed patient who was diagnosed as having drug resistant bacteria, showed *M. leprae* in his tissue with resistance to the drug. Even an untreated patient showed primary resistance to the drug (table 3).

It is clear that *M. leprae* are capable of taking up uracil and using it as a metabolite to some extent as indicated previously by Wheeler *et al.* (1984). The question that arose was, does this happen inside a host cell? If so, is there a modification in the level of incorporation? Thus comparison was made between the total uptake of uracil by free *M. leprae* in suspension and *M. leprae* phagocytosed by macrophages. The experimental data provided in table 5 clearly showed an equal amount of *M. leprae* could have higher level of incorporation inside the host cells than as a free suspension. This could be due to some modification or activation of the metabolism of *M. leprae* inside the cells which facilitated increased uptake. This could also account for the consistent uptake of thymidine with fewer bacteria inside the macrophages (Prasad *et al.*, 1981; Nath *et al.*, 1982) as compared to poor or no uptake of thymidine by greater number of bacteria as free cell suspension (Khanolkar *et al.*, 1978). Even though the conditions of treatment of free *M. leprae* and *M. leprae* inside macrophages appear different while studying the uptake of uracil; they are the most appropriate and optimum conditions for each of the type of the uptake system. Thus a comparison of the system appears justified.

From these observations and that of Nath *et al.* (1982), it is suggested that during early hours of incubation (about 120 h) of *M. leprae*, there is an activation of the metabolism of the bacteria inside the macrophages.

It is thus clear that uracil can be used as a metabolic marker for determining viability, monitor maintenance of the viability and drug sensitivity of *M. leprae* while they are inside the macrophages and also to some extent while they are in free

suspension. The advantage of determining viability inside the macrophages would be that one could determine the effect of immunomodulators as well. This is important in a disease like leprosy in which the patients have poor or no cell mediated immunity. We have recently used macrophages phagocytosed *M. leprae* and uracil thymine to show effect of immunomodulation by lymphokines, and immunostimulants on the viability of *M. leprae* (data under publication).

## Acknowledgements

The authors wish to acknowledge the help received from Acworth Leprosy Hospital, Bombay in the supply of human materials and Dr. E. Storrs and LEPRAs, UK for help with the supply of infected tissues from armadillo. Financial support under PL-400 from National Institute of Health, USA is gratefully acknowledged.

## References

- Ambrose, E. J., Khanolkar, S. R. and Chullawalla, R. G. (1978) *Lep. India*, **50**, 131.
- Bloom, B. R. and Mehra, V. (1984) *Immunol. Rev.*, **80**, 28.
- Day, G. A. (1960) *Anal. Biochem.*, **1**, 271.
- Quickshank, R. (1968) *Medical Microbiology*, (Living Stone: E and S).
- Strople, A. M. and Hanks, J. H. (1980) *Int. J. Lep.*, **49**, 59.
- Wutz, D. J. and Cline, M. J. (1972) *J. Infect. Dis.*, **125**, 416.
- Khanolkar, S. R., Ambrose, E. J., Chullawalla, R. J. and Bapat, C. V. (1978) *Lep. Rev.*, **49**, 187.
- Khanolkar, S. R. and Wheeler, P. R. (1983) *F.E.M.S. Microbiol. Lett.*, **20**, 273.
- Khanolkar, M. V., Jagannathan, R. and Mahadevan, P. R. (1984) *J. Biosci.*, **6**, 709.
- Nath, I., Prasad, H. K., Sathish, M., Shreevastava, K. V., Sheshadri, P. S. and Iyer, C. G. S. (1982) *Antimicrob. Agric. Chemother.*, **21**, 26.
- Prasad, H. K. and Nath, I. (1981) *J. Med. Microbiol.*, **14**, 279.
- Strople, G. A. W. and Rainbow, S. (1981) *Ann. Immunol. (Inst. Pasteur)*, **132D**, 281.
- Strople, G. A. W., Champion, B. R., Steele, J., Varey, A. M. and Stanford, J. L. (1985) *Clin. Exp. Immunol.*, **59**, 414.
- Shetty, V. P., Mehta, L. N., Irani, P. R. and Antia, N. H. (1980) *Lep. India*, **52**, 5.
- Shah, L., Talati, S., Mahadevan, P. R. (1983) *J. Biosci.*, **5**, 235.
- Wheeler, P. R. (1984) *Int. J. Lep.*, **52**, 208.



## ***in vitro* effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases**

N. GIRIDHARAN†, MAHTAB S. BAMJI and M. N. MADHYASTHA\*

National Institute of Nutrition, Indian Council of Medical Research, Jamai Osmania, Hyderabad 500 007, India

\*Department of Postgraduate Studies and Research in Biosciences, University of Mangalore, Mangalore 574 152, India

**Abstract.** The *in vitro* inhibition of several rat testis dehydrogenases by gossypol was examined. Inclusion of the coenzyme (substrate for NADP<sup>+</sup>-isocitrate dehydrogenase) in the preincubation mixture containing the enzyme and gossypol, protected the enzymes against inhibition by gossypol. Lactic dehydrogenase-X was amongst the least protected enzymes. This, coupled with its low  $K_i$  for gossypol makes it one of the most vulnerable target enzymes *in vivo* for gossypol action.

The inhibition kinetics for lactic dehydrogenase-X were competitive when NADH was present during preincubation, but non-competitive when the coenzyme was excluded during preincubation. In the latter condition, the enzyme seems to undergo progressive inactivation with time causing a nonreversible type of inhibition.

**Keywords.** Gossypol; lactic dehydrogenase-X; malate dehydrogenase; testicular dehydrogenases.

## **Introduction**

Gossypol is a potent antifertility agent for males of a variety of species (Sang, 1983). The mechanism of action at molecular level, however, remains speculative. Recent studies from our laboratory and others (Giridharan *et al.*, 1982; Lee *et al.*, 1982; Tso and Lee, 1982) show that sperm-specific lactic dehydrogenase-X (EC 1.1.1.27) is inhibited by gossypol and this effect may be causally related to the antifertility action of gossypol. This enzyme is involved in sperm metabolism and provides energy for sperm motility (Burgos *et al.*, 1982; Hutson *et al.*, 1977).

Gossypol, has also been found to inhibit other forms of lactic dehydrogenase and other enzymes, (Lee *et al.*, 1982; Lee and Mallings, 1981; Tso *et al.*, 1982; Oligati *et al.*, 1984), suggesting that its effect on sperm lactic dehydrogenase-X may be non-specific. There is considerable controversy in the literature regarding the characteristics of inhibition of lactic dehydrogenase-X by gossypol. While some workers found the inhibition to be reversible (Giridharan *et al.*, 1982; Oligati and Toscano, 1983; Burgos *et al.*, 1986), others found it to be non-reversible (Lee *et al.*, 1982).

The studies reported in this paper were aimed at examining the effects of gossypol on lactic dehydrogenase-X and some other key testicular dehydrogenases, under different conditions of enzyme assay, to clarify the controversies existing in the

### Enzyme preparation

For all studies described below, adult Wistar/NIN strain of rats were used. The testes from these animals were homogenized in 9 volumes of 0.25 M sucrose using polytron and the homogenate was subjected to differential centrifugation at 0–4°C. The 100,000 *g* supernatant without any further treatment was used for assaying the following testis dehydrogenases: lactic dehydrogenase-X, sorbitol dehydrogenase (EC 1.1.1.14),  $\alpha$ -glycerophosphate dehydrogenase (EC 1.1.1.8), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), NADP<sup>+</sup>-isocitrate dehydrogenase (EC 1.1.1.42) and malate dehydrogenase (EC 1.1.1.37). Apart from these, lactic dehydrogenase-1 and 5 were also examined in the cytosolic preparation from rat heart and muscle, respectively.

For measurement of kinetic constants, lactic dehydrogenase-X was partially purified from 100,000 *g* supernatant by heat treatment and acidification with 1 N acetic acid, as described by Schatz and Seagal (1969). This 'heat-treated' preparation was readjusted to pH 7.3 for enzyme assay. It showed 3 fold increase in specific activity compared to the untreated supernatant.

### Gossypol preparation

Purified cotton-seed gossypol was gifted by Dr. A. V. B. Sankaram, Regional Research Laboratory, Hyderabad (Giridharan *et al.*, 1982).

### Enzyme assays

Lactic dehydrogenase-X was assayed by the method of Schatz and Seagal (1969) using a final concentration of 4.1 mM  $\alpha$ -ketoglutarate and 0.17 mM NADH in the assay mixture. Rest of the testicular dehydrogenases were assayed as described by Shen and Lee (1976). The final concentration of the substrate and the coenzyme for these enzyme assays were as follows: 66.6 mM fructose and 0.19 mM NADH for sorbitol dehydrogenase, 0.78 mM dehydroxyacetone phosphate and 0.22 mM NADH for  $\alpha$ -glycerophosphate dehydrogenase, 3.33 mM glucose-6-phosphate and 0.19 mM NADP for glucose-6-phosphate dehydrogenase, 0.5 mM isocitrate and 0.13 mM NADP for isocitrate dehydrogenase and 0.5 mM oxaloacetic acid and 0.2 mM NADH for malate dehydrogenase. Lactic dehydrogenase-1 and 5 were assayed as described by Bergmeyer *et al.* (1974) using a final concentration of 0.6 mM pyruvate and 0.18 mM NADH in the assay mixture.

All activity measurements were done in Gilford spectrophotometer at 340 nm and 37°C. Enzyme velocity was expressed as changes in absorbance per min ( $\Delta$ absorbance/min).

For inhibition studies, alcoholic solution of gossypol was used. The solvent, alcohol at the concentration used in the study had no effect on the activity of the enzymes studied. The preincubation of enzymes with gossypol in the presence or absence of either the substrate or the coenzyme was done under conditions described

hydrogenase were calculated as described by Segel (1975) and Dixon and Webb (1979). For these measurements, the range of NADH concentration used was 0.05–0.2 mM and 0.01–0.2 mM, respectively, for lactic dehydrogenase-X and malate dehydrogenase.

## Results

### *Effect of inclusion of substrate or coenzyme in the preincubation mixture containing coenzyme and gossypol*

In the absence of both the substrate as well as the coenzyme in the preincubation mixture, all the enzymes were markedly inhibited by gossypol. Under this condition the inhibition was most marked for glucose-6-phosphate dehydrogenase ( $IC_{50}$  8  $\mu$ M) and least for sorbitol dehydrogenase ( $IC_{50}$  > 80  $\mu$ M). Inclusion of coenzyme in the preincubation mixture protected all the enzymes (except isocitrate dehydrogenase) against gossypol inhibition. In the case of isocitrate dehydrogenase, isocitrate conferred greater protection ( $IC_{50}$  > 80  $\mu$ M) than NADP ( $IC_{50}$  36  $\mu$ M). Apart from the coenzyme, glucose-6-phosphate dehydrogenase was protected by its substrate as well. Amongst the dehydrogenases studied, the coenzyme-mediated protection was least for malate dehydrogenase and lactic dehydrogenase-X, being 29 and 43%, respectively (figure 1). Lactic dehydrogenase 1 and 5 were also protected against inhibition with gossypol in the presence of NADH in the preincubation mixture.

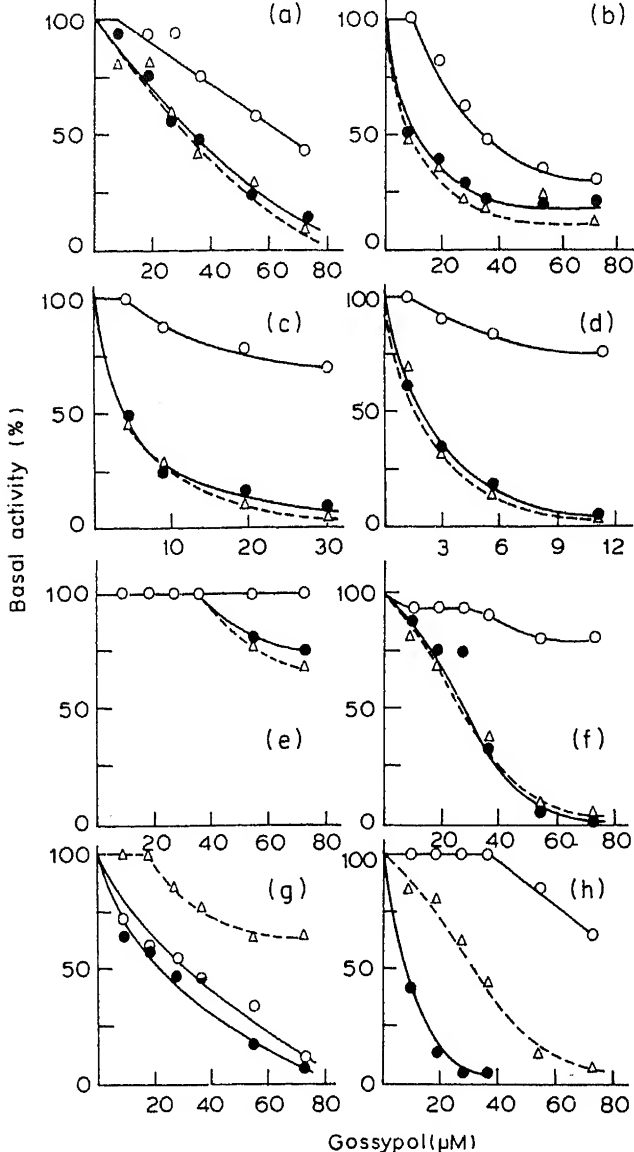
### *Effect of NADH in the preincubation mixture on the kinetic properties of lactic dehydrogenase-X and malate dehydrogenase*

The data in table 1 suggest that when NADH was present in the preincubation mixture, the inhibition characteristic of lactic dehydrogenase-X was of the reversible competitive type, where  $V_{maxapp}$  was not altered but the  $K_{mapp}$  was increased. On the other hand, when NADH was not present in the preincubation mixture and the reaction was started with NADH, the inhibition acquired a non-competitive character where  $K_{mapp}$  was unaffected but  $V_{maxapp}$  was reduced. Similar trends were observed for malate dehydrogenase as well.

Values for  $K_{iapp}$  for the enzymes under conditions where NADH was omitted from the preincubation mixture, were erratic and hence not reported. In the presence of NADH, the  $K_{iapp}$  of lactic dehydrogenase-X and malate dehydrogenase were 1.94 and 5.3  $\mu$ M, respectively.

### *Effect of enzyme concentration and duration of preincubation on the inhibition of lactic dehydrogenase-X by gossypol*

In this experiment, the activity of lactic dehydrogenase-X at different concentrations of the enzyme and different times of preincubation, in the presence and absence of coenzyme was measured, using a fixed concentration of 10  $\mu$ M gossypol.



**Figure 1.** Effect of inclusion of substrate or coenzyme in the preincubation mixture containing enzyme and gossypol.

(a), Lactic dehydrogenase-X; (b), malate dehydrogenase; (c), lactic dehydrogenase-1; (d), lactic dehydrogenase-5; (e), sorbitol dehydrogenase; (f),  $\alpha$ -glycerophosphate dehydrogenase; (g), isocitrate dehydrogenase; (h), glucose-6-phosphate dehydrogenase.

Buffered solutions of the enzymes and varying concentrations of gossypol were preincubated with, (●) neither substrate nor coenzyme, (Δ) only substrate, (○) only coenzyme, for 15 min and the reactions initiated appropriately. The concentration of the substrate and coenzyme were as described in 'materials and methods' section. The protein content per 100  $\mu$ l of the cytosol in the assay mixture were a, e, f, g and h, 400–440  $\mu$ g; b, 44  $\mu$ g; c and d, 3  $\mu$ g. Inhibition of the enzyme activity was expressed as percentage of

**Table 1.** Effect of NADH in the preincubation mixture on the  $V_{\max}$  and  $K_m$  for lactic dehydrogenase-X.

NADH in the preincubation mixture	Concentrations of gossypol ( $\mu\text{M}$ )	Kinetic constants	
		$V_{\max}$ ( $\Delta\text{absorbance}$ )	$K_m$ (mM) NADH
Present	0.0	0.049	0.0051
	0.625	0.050	0.0061
	1.25	0.050	0.0073
	2.5	0.049	0.0083
	5.0	0.049	0.012
	10.0	0.050	0.027
	20.0	0.032	0.029
Absent	0.0	0.057	0.004
	0.625	0.049	0.0039
	1.25	0.047	0.004
	2.5	0.041	0.004
	5.0	0.034	0.0035
	10.0	0.016	0.0036
	20.0	0.008	0.0038

The protein content in the assay mixture for the enzyme preparation was 32  $\mu\text{g}$ .

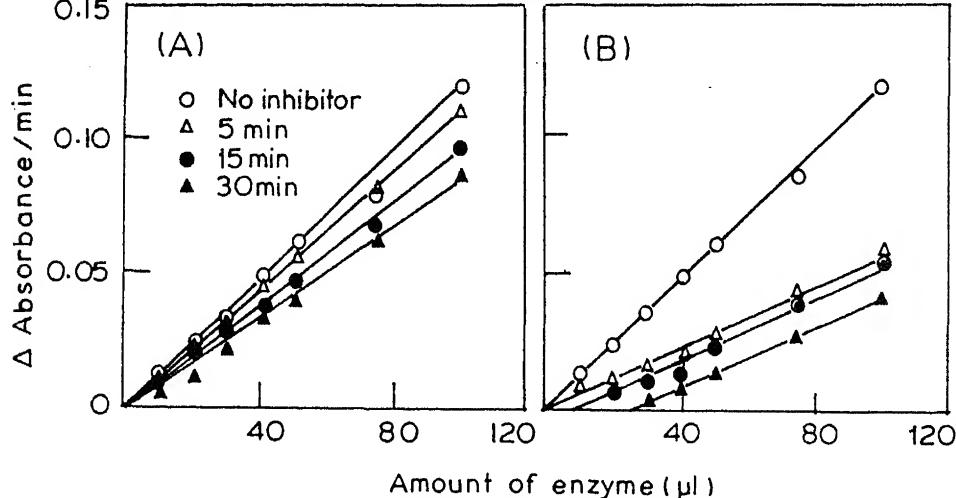
The data in figure 2A show that in the presence of NADH, the plot of enzyme concentration against enzyme activity passed through the origin, regardless of the duration of the preincubation of the enzyme with the inhibitor, suggesting a typical reversible type of inhibition. However, by omitting the coenzyme from the preincubation mixture, the inhibition characteristics appeared to be non-reversible type, wherein the plots of varying concentrations of the enzyme against enzyme activity did not pass through the origin, except, when the duration of preincubation was very short (figure 2B).

The duration of preincubation had no effect on the enzyme activity without the inhibitor and hence only a representative plot is presented in the figure 2.

## Discussion

An earlier suggestion of Lee *et al.* (1982) that the inhibition of sperm-specific lactic dehydrogenase-X by gossypol may be the basic molecular mechanism of gossypol-mediated infertility in males was questioned recently, when it was observed that other enzymes are also inhibited by gossypol. The data presented here show that although a variety of dehydrogenases are indeed inhibited by gossypol in the absence of the coenzyme and substrate in the preincubation mixture, the inhibition was markedly reduced by including the substrate in the preincubation mixture of lactic dehydrogenase and coenzyme for all the other dehydrogenases. Judging from the extent of coenzyme mediated protection, lactic dehydrogenase-X





**Figure 2.** Effect of enzyme concentration and duration of preincubation on the inhibition of lactic dehydrogenase-X by gossypol.

(A), Buffered solution of the enzyme were preincubated with  $10\ \mu\text{M}$  gossypol and  $0.17\ \text{mM}$  NADH for the times indicated in the graph. At the end of each time point, reaction was started with  $4.1\ \text{mM}$ ,  $\alpha$ -ketoglutarate; (B), buffered solution of the enzyme was preincubated with  $10\ \mu\text{M}$  gossypol and  $4.1\ \text{mM}$   $\alpha$ -ketoglutarate for times indicated in the graph. At the end of each time point, reaction was started with  $0.17\ \text{mM}$  NADH.

The protein content of the heat-treated lactic dehydrogenase-X preparation in the assay mixture was  $32\ \mu\text{g}$ .

than that of lactic dehydrogenase-X. This may make lactic dehydrogenase-X a more vulnerable target for gossypol action.

The marked controversy in the literature regarding the type of inhibition of lactic dehydrogenase-X with gossypol (reversible or irreversible) appears to be due to the conditions of preincubation. A true reversible inhibitor is expected to show same inhibition characteristics regardless of the sequence of addition. But the data presented in table 1 show that by omission of NADH from the preincubation mixture, the inhibition pattern changes from competitive to non-competitive type.

Segel (1959) has suggested using plots of varying enzyme concentration against enzyme activity in the presence or absence of the inhibitor to distinguish between reversible and irreversible inhibition. The data in figure 2 suggest that in the absence of NADH in the preincubation mixture, the inhibition of lactic dehydrogenase-X by gossypol is non-reversible.

Apart from the presence or absence of NADH, the duration of preincubation also influences the characteristics of the inhibition (figure 2). At the shorter time interval of 5 min, the inhibition was reversible, regardless of the presence or absence of the coenzyme in the preincubation mixture. This would explain the observations of Oligati and Toscano (1983). The irreversible inhibition seen by Lee *et al.* (1982) was due to the exclusion of the coenzyme from the preincubation mixture and prolonged period of 20 min preincubation.

In general, from the data presented here and other workers, it appears that

ation of promethazine is less than 5 min. The enzyme is possibly inactivated with ne by gossypol or some breakdown products of gossypol, causing a non-reversible type of inhibition. NADH is able to prevent this partially by reducing the velocity instant of the reaction as suggested by Dixon and Webb (1979) for some irreversible inhibitors. Under such conditions the inhibitor combines with the substrate-binding group at the active centre, producing a competitive effect similar to a reversible type inhibition.

## Acknowledgement

The authors are grateful to Dr. B. S. Narasinga Rao, Director, National Institute of Nutrition, Hyderabad, for his keen interest and encouragement through-out the period of this study.

## References

- Bergmeyer, H. U. and Bernt, E. (1974) *Methods Enzym. Anal.*, **2**, 574.
- Burgos, C., Coronel, C. E., Grez de Burgos, N. M., Rovai, L. E. and Blanco, A. (1982) *Biochem. J.*, **208**, 413.
- Burgos, C., Grez De Burgos, N. M., Rovai, L. E. and Blanco, A. (1986) *Biochem. Pharmacol.*, **35**, 801.
- Dixon, M. and Webb, E. C. (eds) (1979) in *Enzymes* (London: Longman Group Ltd.) p. 47.
- Idharan, N., Bamji, M. S. and Sankaram, A. V. B. (1982) *Contraception*, **26**, 607.
- Johnson, S. M., Vandop, C. and Lardy, H. A. (1977) *J. Biol. Chem.*, **252**, 1309.
- Lee, C. Y. and Malling, H. V. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **40**, 2790.
- Lee, C. Y. G., Moon, Y. S., Yuan, J. H. and Chen, A. F. (1982) *Moll. Cell. Biochem.*, **47**, 65.
- Wry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Agati, K. L., Hoffer, A. P. and Toscano, W. A. Jr. (1983) *Biochem. Biophys. Res. Commun.*, **115**, 180.
- Agati, K. L., Toscano, D. G., Atkins, W. M. and Toscano, W. A. Jr. (1984) *Biochem. Biophys.*, **231**, 411.
- Atz, L. and Seagal, H. L. (1969) *J. Biol. Chem.*, **247**, 2044.
- gel, I. H. (1975) in *Enzyme kinetics-Behaviour and analysis of rapid equilibrium and steady state enzyme systems* (New York: John Wiley and Sons) p. 100.
- ng, G. W. (1983) in *Hormones in Normal and Abnormal Human Tissues* (eds K. Fotherby and S. B. Pal) (New York: Walter de Gruyter and Co.) vol. 3, p. 215.
- en, R. S. and Lee, I. P. (1976) *J. Rep. Fertil.*, **48**, 301.
- o, W. W. and Lee, C. S. (1982) *Int. J. Androl.*, **5**, 205.
- o, W. W., Lee, C. S. and Tso, Y. W. (1982) *Arch. Androl.*, **9**, 31.



# Conformational and ion-binding properties of cyclolinopeptide A isolated from linseed

D. CHATTERJI, M. B. SANKARAM† and

D. BALASUBRAMANIAN\*

Centre for Cellular and Molecular Biology, Regional Research Laboratory Campus, Hyderabad 500 007, India

†Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, West Germany

**Abstract.** The conformation of the cyclic nonapeptide from linseed, cyclolinopeptide A in methanol and in acetonitrile has been elucidated by one- and two-dimensional nuclear magnetic resonance. The molecule is folded in a  $\beta$ -turn conformation. Cyclolinopeptide A interacts and weakly complexes with  $Tb^{3+}$  (a  $Ca^{2+}$  mimic ion) with the metal ion positioned proximally to the Phe residue, but with no substantial structural alteration upon metal binding. Cyclolinopeptide A is also seen to aid the translocation of  $Pr^{3+}$  (another  $Ca^{2+}$  mimic) across unilamellar liposomes. However, cyclolinopeptide A does not phase transfer or act as an ionophore of calcium ion itself. Experiments using lanthanide ions thus do not necessarily indicate any ionophoretic ability of the complexone towards calcium ions.

**Keywords.**  $\beta$ -Turn and calcium ion binding; one- and two-dimensional nuclear magnetic resonance of peptides; ionophoretic assay; lanthanide probes; fluorescence energy transfer.

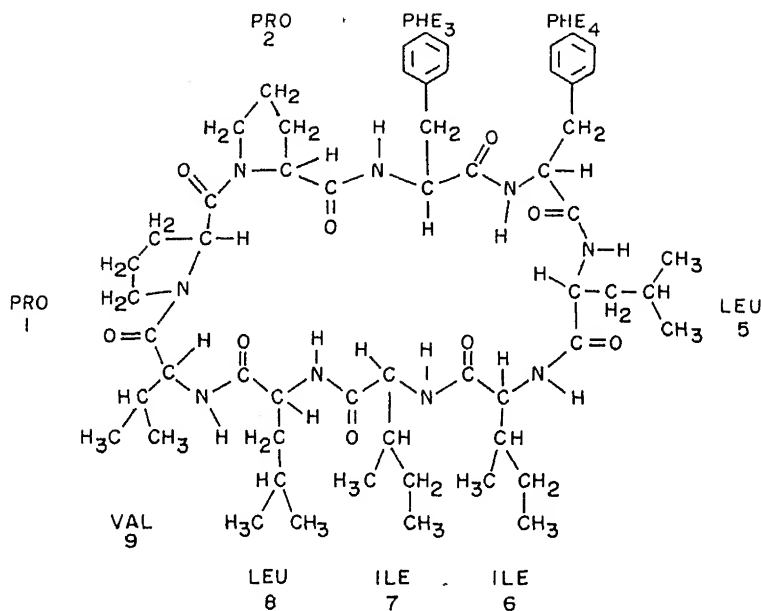
## Introduction

Cyclolinopeptide A (CLPA) is a homodetic cyclic nonapeptide from linseed (Kaufmann and Tobschirbel, 1959) and has the structure I. Its partial homology with the ionophore antamanide (structure II) has evoked interest in the conformation, ionophoretic properties and the biological role of CLPA. Early work on its spectral and conformational analysis (Naider *et al.*, 1971; Brewster and Bovey, 1971; Tonelli, 1971) suggested a flexible conformation for CLPA in solution. Siemion *et al.* (1977) had suggested that CLPA might adopt a  $\beta$ -turn conformation. They also found the peptide to complex weakly with  $K^+$  ions, with little conformational change upon ion binding. Our own studies around the same time (Balasubramanian *et al.*, 1976) showed that CLPA adopts 3 broad classes of conformations (termed A, B and C) in solution, binds  $Ca^{2+}$  ions weakly, and changes from the B conformation into the A form upon binding this ion in methanol.

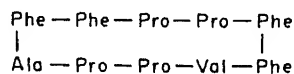
We present here results of our further studies on the conformation of CLPA and its ion binding propensities. This is motivated particularly by the growing evidence for the suggestion that the  $\beta$ -turn region of a peptide chain offers itself as a favourable ligand for the binding of  $Ca^{2+}$  and like ions (Vogt *et al.*, 1979; Ananthanarayanan *et al.*, 1985), and it would be worthwhile testing this suggestion with CLPA. In this context, we have identified the  $\beta$ -turn region in CLPA using the method of one- and

\*To whom all correspondence should be addressed.

Abbreviations used: CLPA, Cyclolinopeptide A; NMR, nuclear magnetic resonance; ULV, unilamellar



I (CLPA)



II

(ANTAMANIDE)

two-dimensional nuclear magnetic resonance (1D and 2D NMR), located the site of metal ion interaction in the peptide, using the fluorophore  $\text{Tb}^{3+}$  which is an excellent  $\text{Ca}^{2+}$  mimic (Brittain *et al.*, 1976; Horrocks *et al.*, 1984) and monitored the ion-translocating ability of CLPA across unilamellar lipid vesicles (ULV) using  $\text{Pr}^{3+}$  ion, another  $\text{Ca}^{2+}$  mimic (Sankaram, 1983; Sankaram *et al.*, 1985). However  $\text{Ca}^{2+}$  does not appear to bind to CLPA nor does it get translocated across ULVs by the peptide. The ions  $\text{Tb}^{3+}$  and  $\text{Pr}^{3+}$  also bind only weakly to CLPA, essentially as outer sphere complexes. It thus appears that while these ions may mimic  $\text{Ca}^{2+}$ , they display special properties such as outer sphere complexation (which  $\text{Ca}^{2+}$  cannot), and hence the use of these ions for assaying the  $\text{Ca}^{2+}$  ionophoretic activity is not fool-proof.

## Materials and methods

CLPA was isolated from linseed cake by Soxhlet extraction from acetone and recr-

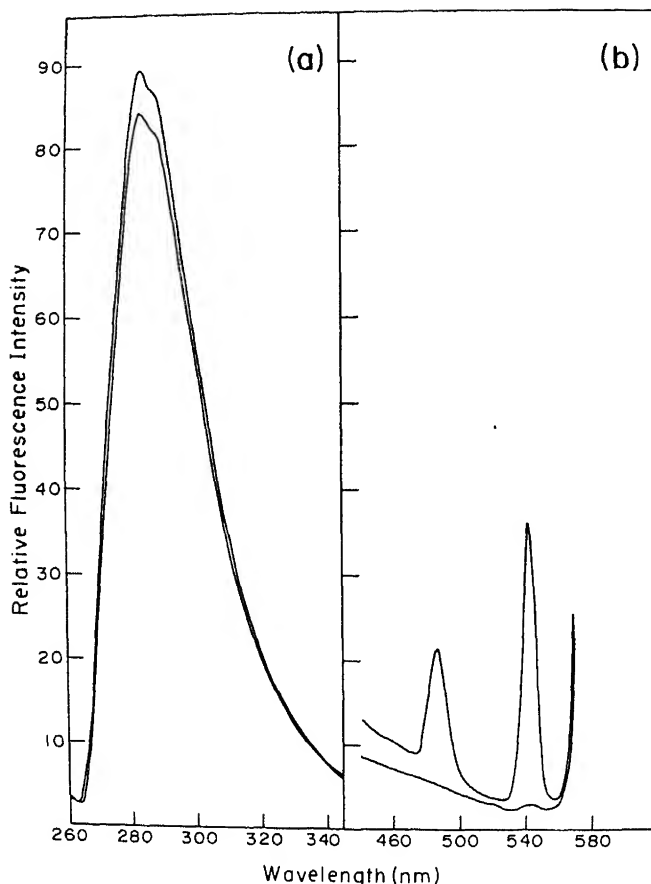
recorded on a 650-10S Hitachi spectrofluorimeter, with care taken to avoid the inner filter effect.  $[^1\text{H}]$ -NMR spectra were obtained using a Bruker 500 MHz FT NMR spectrometer. Two dimensional J-resolved correlation spectroscopy (COSY) was done at 24°C following the standard pulse sequence discussed in detail elsewhere (Nagayama *et al.*, 1977; Benn and Gunther, 1983; Redfield and Kunz, 1979). The spectral width was 8064-516 MHz. 32 free induction decays were accumulated for each value of  $t_1$  with 400 experiments or  $t_1$  values in the time domain. For the nuclear Overhauser effect (NOE) experiments, a given peak of interest was selectively saturated by gated irradiation at that frequency, by using just enough decoupler power to almost saturate the resonance in 0.1 s. The NOE data were collected directly by interleaving, as NOE difference spectra, by using difference Fourier techniques (Tropp and Redfield, 1981). Five pulses irradiated on the resonance line and the same number of pulses irradiated off resonance were interleaved in an alternating cycle, with memory negation, to generate the difference free induction decay which was Fourier transformed to generate the difference spectrum in the frequency domain.

## Results and discussion

### *Fluorescence energy transfer between CLPA and $\text{Tb}^{3+}$*

$\text{TbCl}_3$  displays an inherent fluorescence emission spectrum with characteristic maxima at 485 nm and 540 nm, when excited at 290 nm but only when present at sufficiently large concentrations. However, at a concentration below 1 mM in methanol or in acetonitrile, no fluorescence signals were observed at the above region indicating thereby that the enhancement of  $\text{Tb}^{3+}$  fluorescence at this concentration range could be conveniently studied if it binds to the peptide. The fluorescence spectrum of CLPA itself in MeOH showed a characteristic emission band at 284 nm when excited at 257 nm, typical of the phe moiety in the peptide. The matching of the emission band of CLPA and the excitation band of  $\text{Tb}^{3+}$  makes it possible to monitor any energy transfer between the peptide and the ion and to investigate the binding process if that occurs. Figure 1 shows the enhancement of the 540 nm emission band of  $\text{Tb}^{3+}$  and the concomitant quenching of the 284 nm band of the peptide that occur as  $\text{TbCl}_3$  is added to a solution of CLPA in methanol. Since the fluorescence intensity of the phe moieties in CLPA is orders of magnitude more than that of the CLPA :  $\text{Tb}^{3+}$  system, any energy transfer would be expected to only negligibly affect the phe emission band at 284 nm. However, the phe-sensitized emission in  $\text{Tb}^{3+}$  is seen to be quite appreciable at 540 nm, which results in a small but significant drop in the peptide emission at 284 nm. It appears that the maximal fluorescence change could be observed at a mol ratio of 5:1 CLPA :  $\text{TbCl}_3$ . Also, identical results were obtained with acetonitrile as the solvent. It has been reported earlier that the conformation of CLPA is the same in acetonitrile and in alcohols (Balasubramanian *et al.*, 1976).

It is noteworthy that the fluorescence of  $\text{Tb}^{3+}$  is substantially enhanced in the



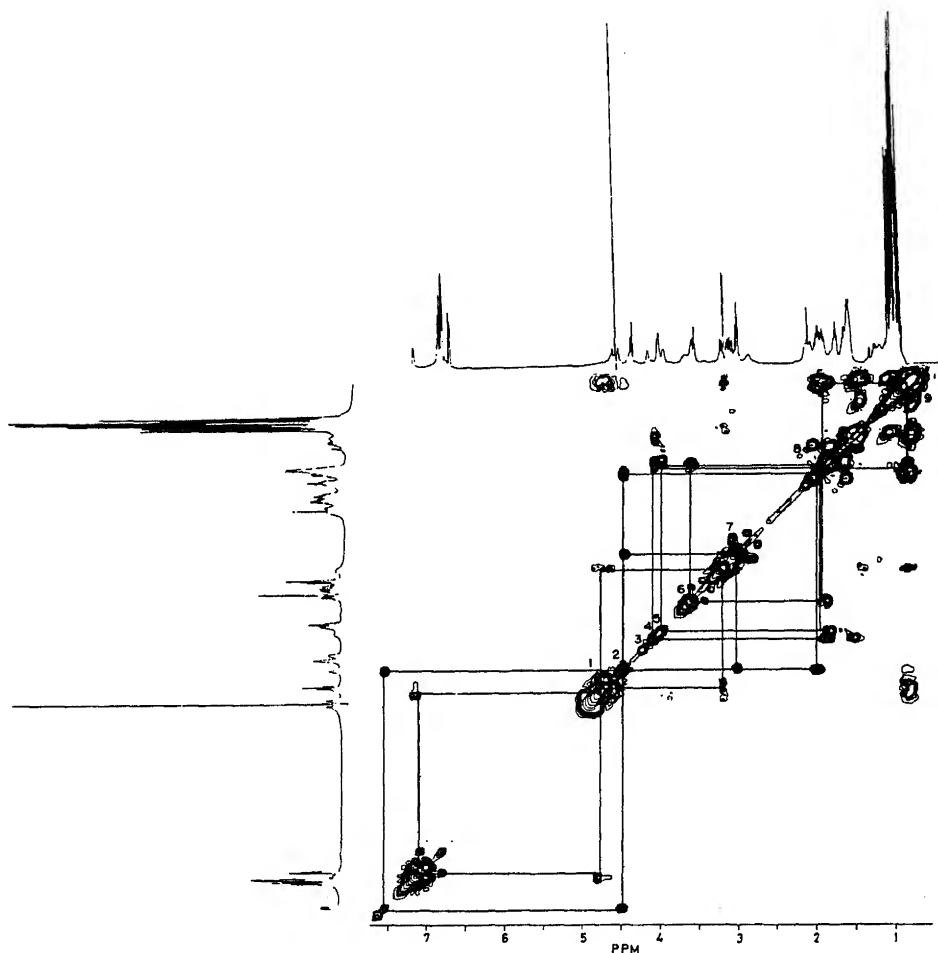
**Figure 1.** (a), Fluorescence spectrum of CLPA, upon excitation at 257 nm. The top curve is for the free peptide in methanol (1 mM concentration) and the lower curve is for the system CLPA: TbCl<sub>3</sub> (5:1 mol ratio). (b), The emission spectrum of TbCl<sub>3</sub> (0.2 mM) in methanol, in the presence and absence of CLPA. Excitation wavelength 290 nm. The lower curve shows the spectrum of TbCl<sub>3</sub> alone while the upper curve is for the system CLPA: TbCl<sub>3</sub> (5:1 mol ratio). The peptide itself does not fluoresce in this region.

intensity in the 285–290 nm region (the emission region of the peptide) makes any dipole-dipole or Forster type energy transfer unlikely; instead, a close proximity between the Tb<sup>3+</sup> ion and the phe residues of CLPA, *i.e.*, binding of the ion, is indicated.

Though substantial spectral changes occur when TbCl<sub>3</sub> is added to CLPA, measurements of the stability constant of the complexation and its stoichiometry either by fluorimetry or electrical conductivity showed very weak complexation and no definite stoichiometry between TbCl<sub>3</sub> and CLPA. This behaviour of CLPA is akin to

*Conformational features by NMR spectroscopy*

The fluorescence results and our earlier circular-dichroism results (Balasubramanian *et al.*, 1976) suggest that CLPA displays detectable spectral changes in the presence of certain ions, but it is not clear whether these changes reflect conformational alterations in the molecule or are due to a medium effect. In order to investigate these possibilities further, we have used 1- and 2-D NMR spectral methods. Figure 2 shows the J-resolved correlation spectrum (2D-COSY) of CLPA in the solvent



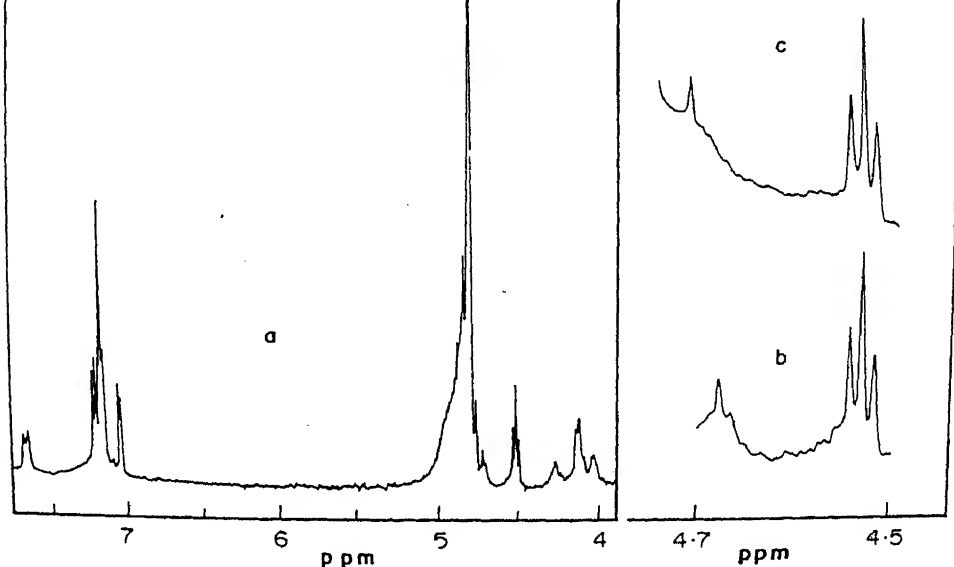


CD<sub>3</sub>OD. This solvent was used since all exchangeable protons in CLPA would be deuterated here, thereby simplifying the spectrum. In the assignment of the peaks, we have been aided by the earlier work (Brewster and Bovey, 1971; Siemion *et al.*, 1977). The  $\delta$ -CH<sub>2</sub> protons of pro residues are the easiest to assign since they stand apart around 3.5 ppm, and are J-coupled to the pro- $\gamma$  protons that occur along with the pro- $\beta$  protons which are in turn coupled to the pro- $\alpha$  CH. The latter will of course not be coupled at any NH protons. Thus, one of the cross peaks in the COSY spectrum at 4.2 ppm is assignable to pro- $\alpha$  CH. The assignment of the signal at 7.55 ppm to a phe-NH proton is also easy since it is correlated to the phe  $\alpha$ -CH proton, which should be the most downfield shifted of the  $\alpha$ -CH manifold in the 4.2–4.8 ppm range. This assignment of the phe-NH and the phe- $\alpha$  CH is consistent with the earlier suggestion that one of these aromatic NH (phe-3) is intramolecularly H-bonded (Siemion *et al.*, 1977), and would not be solvent-exchanged. This was further confirmed by a double irradiation experiment, where selective saturation of the 7.5 ppm signal resulted in a simplification of the  $\alpha$ -CH manifold in the 4.5–4.8 ppm region. Further identification of the phe protons was also achieved by locating the phe- $\beta$  CH<sub>2</sub> through the correlation diagram in figure 2. The second signal below 7.0 ppm region was identifiable as due to Ile-NH, following Siemion *et al.* (1977). All the other assignments, as shown in figure 2, are straight forward and the ambiguities could be resolved by doing 1-D NOE experiments.

Such a 1-D NOE, coupled with double irradiation experiments as well, has suggested that a Val- $\alpha$ -CH occurs close to the phe- $\alpha$ -CH, and is coupled to the val- $\beta$ -CH<sub>2</sub>, and the correlation of the latter to the  $\gamma$ - and these to the  $\delta$ -protons. The leu- $\beta$ -CH that gives rise to a weak cross peak could be assigned as per Brewster and Bovey (1971). The Ile- $\alpha$ -CH occurs just below the pro- $\alpha$ -CH, and could be assigned again following the connectivity diagrams. The assignments are shown in figure 2.

It was earlier surmised (Siemion *et al.*, 1977) that Ile-7-NH is also intramolecularly H-bonded, but its assignment proved difficult due to overlap by the aromatic protons in the 7.1 ppm region (see above). We have been able to clarify and confirm this by using the Tb<sup>3+</sup> induced shifts that occur in the NMR spectrum of CLPA in presence of this ion. Figure 3 shows the spectrum of the system 5:1 CLPA:TbCl<sub>3</sub>. The aromatic region is better resolved here than in free CLPA and there are minor changes upon ion binding, very likely due to the proximal interaction between the ion and the phe ring. A sharp multiplet appears between the solvent peak and the  $\alpha$ -CH signals around 4.5 ppm. Now when the signal at 7.7 ppm is irradiated, the multiplicity at 4.8 ppm is reduced into a singlet. Based on the assignment of the 4.8 ppm signal to the Ile- $\alpha$ -CH (see above), the 7.7 ppm signal in the CLPA:TbCl<sub>3</sub> system is assigned to the Ile-NH signal, which has been downfield shifted by the lanthanide ion. Other than this shift, no other changes are seen in the NMR spectrum of CLPA upon the addition of this ion, reflecting the absence of any significant change in the peptide conformation upon Tb<sup>3+</sup> binding. Such would be the case if the ion were to form an outer sphere complex with the peptide. Incidentally, no spectral changes were observed between free CLPA and when Ca<sup>2+</sup> was added to a solution of the peptide, suggesting that Ca<sup>2+</sup> ion is not bound any better than Tb<sup>3+</sup> to CLPA.

We also monitored the isomerization status of the pro residue in CLPA in the free form and in the presence of TbCl<sub>3</sub>. We observed a proton spin-spin coupling

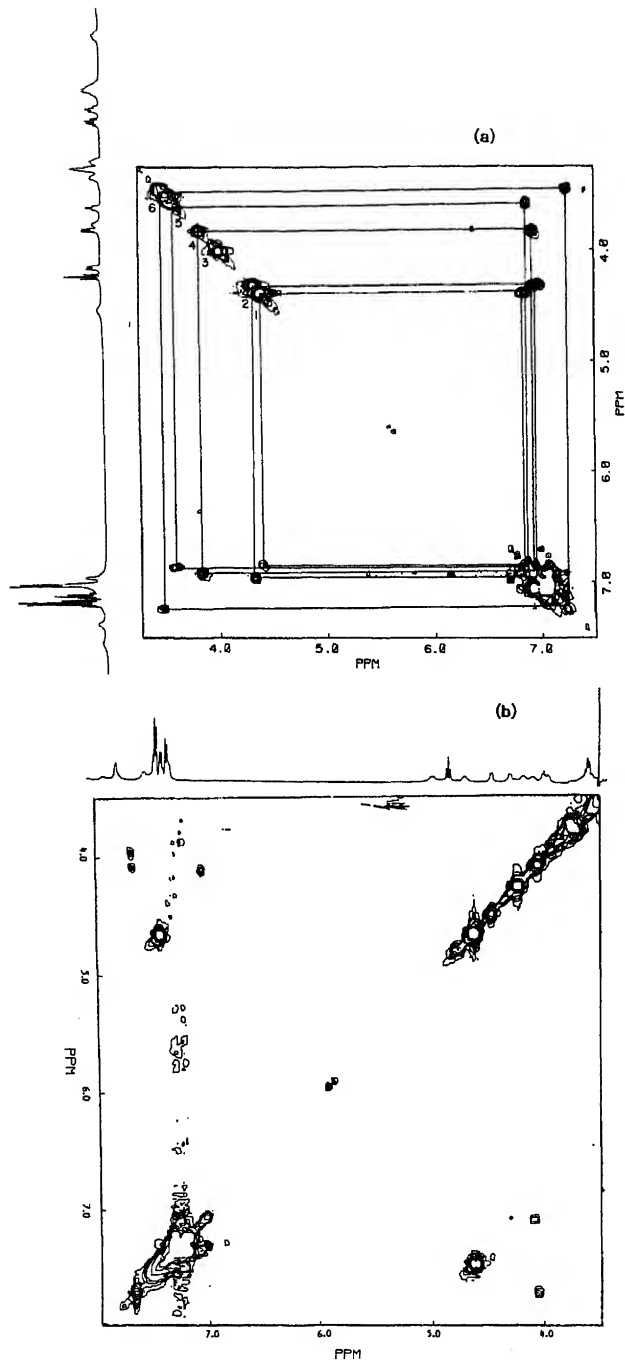


**Figure 3.** 1D-NMR spectrum of CLPA (1 mM) in  $\text{CD}_3\text{OD}$  in the presence of  $\text{TbCl}_3$  (0.3 mM). (a), Downfield region of the system; (b), the  $\alpha$ -CH region; (c), is the  $\alpha$ -CH region with double irradiation at 7.18 ppm.

for at least one of the residues to be in the *cis* (Patel, 1973; Anteunis *et al.*, 1975), is in accordance with the  $^{13}\text{C}$ -NMR data (Siemion *et al.*, 1977), and indicates that this isomer status is unchanged upon  $\text{Tb}^{3+}$  binding. The presence of a *cis* pro residue and the hydrogen bonding through a phe-NH would argue for a  $\beta$ -turn conformation in this region of CLPA.

Further support for this conformational assignment comes from the 2D-COSY spectra of the peptide in the solvent  $\text{CD}_3\text{CN}$  where its conformation is known to be the same as in alcohols. Figure 4a shows that it is possible to resolve and assign all the NH protons of the molecule in this non-exchanging solvent. Such assignment, following the connectivity patterns as in the  $\text{CD}_3\text{OD}$  case, uses the pro- $\delta$ -CH which stands apart between the  $\alpha$ -CH and  $\beta$ -CH regions and which can be connected successively through the neighbouring protons of the residue. The val and phe- $\alpha$ -CH signals around 4.2 ppm and can be individually located since the phe- $\beta$ -CH<sub>2</sub> that connects the latter is the most downfield shifted in its class and has no other connectivity. Therefore, the corresponding NH protons are also easily assigned. Following Brewster and Bovey (1971), one of the Ile-NH should be more downfield shifted, and, as the two Ile- $\alpha$ -CH absorb together, they can be assigned as also their NH protons. The leu protons are then assigned essentially by a process of elimination. All the assignments are indicated in figure 4a for the free peptide.

Upon the addition of  $\text{TbCl}_3$ , the phe- $\alpha$ -CH is downfield shifted compared to the val- $\alpha$ -CH (see figure 4b) and a reduction in the aromatic manifold occurs. While the changes in the phe-NH signal are a little difficult to monitor, the downfield shift of



**Figure 4.** (a), 2D-COSY plot of CLPA (1 mM) in CD<sub>3</sub>CN. The conditions and the display are the same as in figure 2 and the assignments are as under: (1), val-α-CH; (2), phe-α-CH; (3), pro-α-CH; (4), leu-α-CH; (5), Ile-α-CH and (6), Ile-α-CH. The corresponding NH

ppm in the Ile-NH signal upon  $Tb^{3+}$  addition is obvious as in the case of  $_3OD$ .

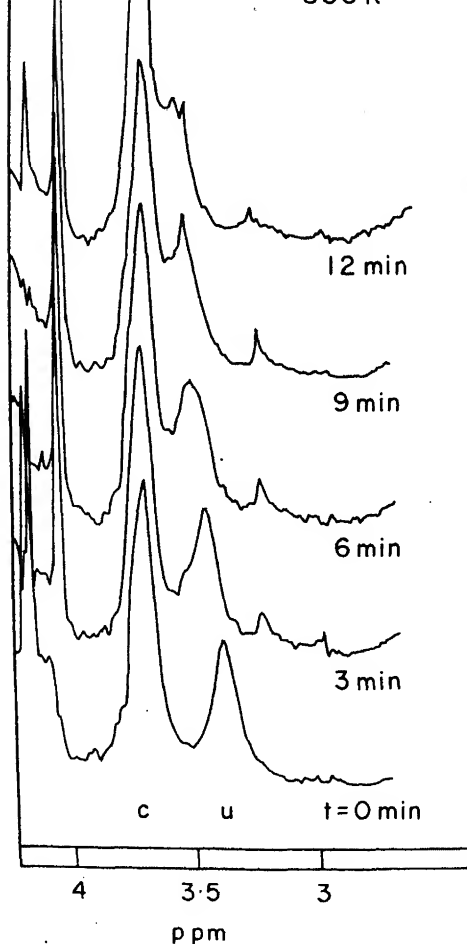
We thus conclude that the conformation of CLPA in these two solvents is the *i.e.* a  $\beta$ -turn conformation with intramolecular hydrogen bonding and a *cis* propeptide. As per earlier evidence, the hydrogen bonding appears to occur *via* the phe-3 and the Ile-7 amide hydrogens as donors.  $Tb^{3+}$  ion appears to bind weakly and the maximal spectral alterations appear to be at a mol ratio of 5:1 CLPA: $TbCl_3$  and the  $Tb^{3+}$  ion appears to be affecting the emission properties of the phe residue through a maximal energy transfer mechanism. Calcium ion does not affect the spectral properties of CLPA to the extent that the calcium ion mimic  $Tb^{3+}$  does. Though  $\beta$ -turn is evident in CLPA, strong complexation with  $Ca^{2+}$  appears absent. Its complexation with  $Tb^{3+}$  appears to be at the surface, as an outer sphere complex involving lone pair electrons of the lanthanide, as in the case of antamanide (Wieland *et al.*, 1972).

#### Lanthanide ion translocation across liposomes by CLPA

We next explored the possibility of whether CLPA can translocate lanthanide ions across a unilamellar vesicle, or a liposome.  $Pr(NO_3)_3$  was the salt chosen since  $Pr^{3+}$  is a well-known mimic of  $Ca^{2+}$  ion (Mikkelsen, 1976) and (ii)  $Pr^{3+}$ , being paramagnetic, would 'lanthanide-shift' the NMR signals of the outer monolayer choline headgroup nuclei at first when added to a suspension of unilamellar vesicles (ULV) of a phospholipid such as dimyristoyl phosphocholine (DMPC); a similar shifting of the inner monolayer choline headgroup NMR signals would occur only when  $Pr^{3+}$  is able to traverse across the ULV bilayer. This then offers a NMR spectral assay of the kinetics of  $Pr^{3+}$  transport across the vesicle, mediated by a transporting agent. The details of such experiments have been given elsewhere (Sankaram, 1983; Sankaram *et al.*, 1985; Hunt, 1980).

In such a kinetic assay on the transport of  $Pr^{3+}$  across a DMPC ULV, mediated by varying mol ratios of CLPA:DMPC revealed that the peptide is indeed able to mediate  $Pr^{3+}$  entry across the liposome (see figure 5 for an illustrative experiment at CLPA:DMPC = 1:17). When the data at various CLPA:DMPC mol ratios were analyzed by means of a plot of log (rate of  $Pr^{3+}$  entry) against log (CLPA concentration), we obtained a stoichiometry of the transporting species to be  $4.8 \pm 0.2$  of CLPA per  $Pr^{3+}$  ion. That CLPA-mediated vesicular fusion does not occur was concluded in experiments involving (i) the addition of CLPA to preformed DMPC vesicles and (ii) the use of preformed vesicles of DMPC + CLPA, and no differences in the kinetic parameters or the NMR signal strengths were seen in the two cases. It thus appears likely that CLPA can translocate  $Pr^{3+}$  ions across unilamellar vesicles, though its efficiency does not seem to compare well with those of valinomycin or gramicidin (Sankaram, 1987, the well-known ionophores (Sankaram *et al.*, 1985). The comparison of CLPA is more apt to the detergents Triton X-100 or the bile salts, which are themselves no strong ion-complexones and yet show multiple stoichiometry in transporting  $Pr^{3+}$  across liposomes (Hunt, 1980; Hunt and Jawaharlal, 1980).

On the other hand, when the ability of CLPA to translocate  $Ca^{2+}$  across



**Figure 5.** Time dependent changes in the NMR signals of the headgroup choline protons of DMPC in preformed ULVs of DMPC:CLPA (mol ratio 17:1) upon the addition of 10 mM  $\text{Pr}(\text{NO}_3)_3$  to the external medium at time  $t=0$ . The ULVs contained 10 mM  $\text{La}(\text{NO}_3)_3$  within, in order to maintain isotonicity. The signal C refers to the outer monolayer protons that are ion-complexed and downfield shifted, while U refers to the inner headgroup protons.  $T=303\text{K}$ , above the phase transition temperature of DMPC. The experimental details are as in Ting *et al.* (1981).

directly and neither can it form an outer sphere complex with the peptide, and thus does not get translocated by CLPA.

The effects seen with the lanthanides are thus not reflective of an ionophoretic ability of CLPA towards group I or II cations, but essentially due to the surface complexation by the lanthanide ions. In light of this, we believe that experiments

ing lanthanide ions does not necessarily indicate any intrinsic  $\text{Ca}^{2+}$ -ionophoretic ability of the transporting agent but only a specialized case of outer sphere effects. Thus, it is questionable whether substances such as the bile salts, Triton X-100, cardiolipin or lysophosphatidyl choline, which are seen in NMR experiments to translocate  $\text{Pr}^{3+}$ , are true ionophores.

*Note added in proof*

We have since done more extensive 2D-NMR and NOE measurements on CLPA in  $\text{D}_2\text{O}$  and  $\text{D}_2\text{Me}_2\text{SO}$  at various temperatures. This has led to some reassignments of signals and also inter-proton distance estimates in the molecule. A detailed paper on the NMR measurements, energy minimization calculations and a model for the conformation of CLPA will soon appear. We are grateful to Professors P. Balaram and Ramakrishnan for their interest that has led to this detailed structural analysis.

## acknowledgements

We thank the Sophisticated Instruments Facility, Indian Institute of Science, Bangalore and the National NMR Facility, Tata Institute of Fundamental Research, Bombay, for NMR spectral time. This work was supported in part by a grant from the Department of Science and Technology, (SERC thrust area programme), New Delhi. We are highly indebted to Dr. S. Rajappa of Hindustan Ciba-Geigy Research Centre, Bombay for interesting us in CLPA, a sample of the peptide and his collaboration in our earlier studies.

## References

- Arnth-Jensen, V. S., Attah-Poku, S. K., Mukkamalla, P. L. and Rehse, P. H. (1985) *J. Biosci.*, **8**, 209.
- Beauchamp, M. J. O., Callens, R. E. A. and Tavernier, D. K. (1975) *Eur. J. Biochem.*, **58**, 259.
- Chakrabarti, D., Chopra, P., Ardesir, F. and Rajappa, S. (1976) *FEBS Lett.*, **65**, 69.
- Conway, R. and Gunther, H. (1983) *Angew. Chem.*, **22**, 350.
- DeWitt, A. I. and Bovey, F. A. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 1199.
- Edwards, H. G., Richardson, F. S. and Martin, R. B. (1976) *J. Am. Chem. Soc.*, **98**, 8255.
- Frederick, Jr., W. D. and Albin, M. (1984) *Prog. Inorg. Chem.*, **31**, 1.
- Frederick, G. R. A. (1980) *FEBS Lett.*, **119**, 132.
- Frederick, G. R. A. and Jawaharlal, K. (1980) *Biochem. Biophys. Acta*, **601**, 678.
- Frederick, H. P. and Tobschirbel, A. (1959) *Chem. Ber.*, **92**, 2805.
- Frederick, R. B. (1976) in *Biological Membranes* (eds D. Chapman and D. F. H. Wallach) (London: Academic Press) p. 153.
- Frederick, R., Mathew, M. K. and Balaram, P. (1980) *FEBS Lett.*, **121**, 365.
- Frederick, K., Wuthrich, K., Bachmann, P. and Ernst, R. R. (1977) *Biochem. Biophys. Res. Commun.*, **78**, 99.
- Frederick, F., Benedetti, E. and Goodman, M. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 1195.
- Frederick, D. J. (1973) *Biochemistry*, **12**, 667.
- Frederick, A. G. and Kunz, S. D. (1979) in *NMR and Biochemistry* (eds S. J. Opella and P. Lu) (New York: Marcel Dekker) p. 225.
- Frederick, M. B. (1983) *Structural, mechanistic and kinetic aspects of Valinomycin mediated transmembrane ion transport*, Ph.D. Thesis, Indian Institute of Science, Bangalore.
- Frederick, M. B., Shastri, B. P. and Easwaran, K. R. K. (1985) *J. Biosci.*, **8**, 343.

- Ting, D. Z., Hagan, P. S., Chen, S. I., Doll, J. D. and Springer, C. S. (1981) *Biophys. J.*, **34**, 189.
- Tonelli, A. E. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 1203.
- Tropp, J. and Redfield, A. G. (1981) *Biochemistry*, **20**, 2133.
- Vogt, H. P., Strassburger, W. and Wollmer, A. (1979) *J. Theor. Biol.*, **76**, 297.
- Wieland, T., Faulstich, H. and Burgermeister, W. (1972) *Biochem. Biophys. Res. Commun.*, **47**, 984.

# Spectroscopic studies on the interaction of bilirubin with symmetrical alkyl diamines

KASTURI LAHIRI PURANAM and P. BALARAM\*

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

**Abstract.** The interactions of symmetrical alkyl diamines with bilirubin-IX $\alpha$  have been examined in dichloromethane and dioxane solutions, by visible region difference spectroscopy and fluorescence methods. In dioxane solutions a clear difference is observed between the complexes of the shorter chain diamines (number of spacer methylene groups ( $n \leq 4$ )) and the longer chain diamines ( $n \geq 6$ ). The variations in spectral features with diamine chain length are less pronounced in dichloromethane. The spectroscopic results are consistent with the occurrence of distinct bilirubin conformations depending upon the solvent and the geometry of the interacting receptor. Based on molecular modelling two conformations are proposed. A 'ridge-tile' model similar to that observed in crystals is favoured for binding to the longer diamines, while a 'quasi-cyclic' structure is preferred for interaction with the short chain diamines.

**Keywords.** Bilirubin conformations; bilirubin-amine interactions; difference spectroscopy; fluorescence.

## Introduction

The structure and interactions of bilirubin, a linear tetrapyrrole pigment (figure 1, presented as the predominant isomer bilirubin-IX $\alpha$ ) formed by the catabolism of hemoglobin, have attracted considerable attention over the past several years (Lightner, 1982; Brodersen, 1980, 1982). Disorders of bilirubin metabolism lead to a variety of clinical syndromes (Berthelot *et al.*, 1982). The physiological importance of bilirubin interactions with plasma albumin, in transport of the pigment, has led to detailed investigations of the interactions of the pigment with albumins from humans and other animal sources (Brodersen, 1982; Blauer *et al.*, 1977). While bilirubin-IX $\alpha$  is an optically inactive molecule, binding to chiral sites on proteins can result in induced circular dichroism, a property that has proved useful for probing pigment-protein interactions (Blauer, 1983; Blauer and Wagniere, 1975). There have however been few attempts to delineate the conformation of bilirubin at the albumin binding

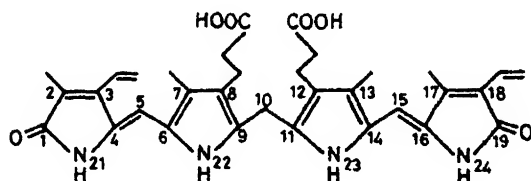


Figure 1. Structure of bilirubin-IX $\alpha$  (4Z, 15Z configuration) with numbering scheme (Lightner, 1982).



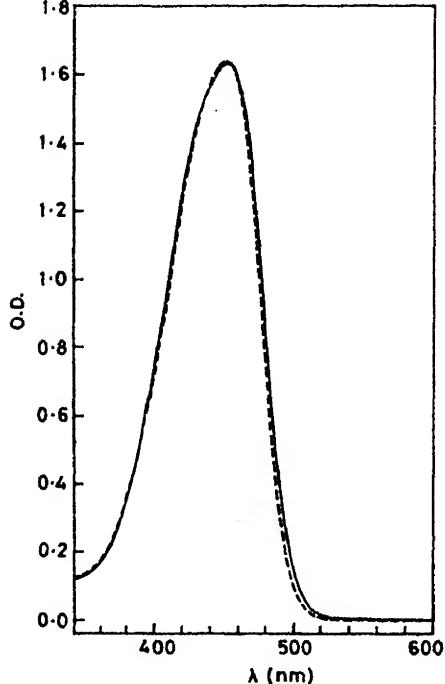
crystalline state (Bonnett *et al.*, 1978). Dramatic changes in the sign of the circular dichroism bands of bilirubin bound to human serum albumin on changing pH or ionic strength (Brodersen, 1982; Blauer, 1983) and the differences in the circular dichroism spectra of the pigment bound to human and bovine albumins suggest that precise orientation of the interacting groups at the receptor site can determine the conformation of the bound pigment (Brodersen, 1982; Blauer, 1983). The presence of two propionic acid sidechains in bilirubin, suggests that electrostatic interactions with positively charged, basic residues may contribute to stabilizing pigment-protein complexes (Marr-Leisy *et al.*, 1985). In this report we describe fluorescence and visible difference spectroscopic studies of the interaction of bilirubin-IX $\alpha$  with symmetrical diamine receptors in organic solvents. The results provide evidence for conformational transitions in bilirubin as a function of the solvent and the nature of the interacting 'receptor' molecule.

## Materials and methods

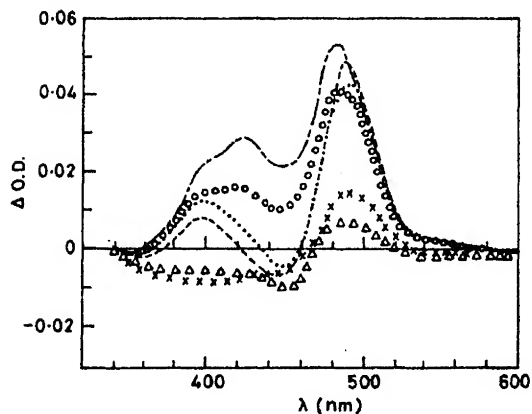
Bilirubin-IX $\alpha$  (Sigma) was purified by a procedure adopted from that of McDonagh and Assisi (1972). Concentrations of bilirubin solutions in organic solvents were estimated using a molar extinction coefficient of  $\epsilon_{455} = 62,000$  (London *et al.*, 1983). 1,2-Diaminoethane (British Drug House), 1,6-diaminohexane (Koch-Light), 1,4-diaminobutane, 1,5-diaminopentane and 1,8-diaminooctane (Sigma) were commercial samples used directly. Dioxane was purified by treatment with concentrated HCl, drying and fractionation (Furniss *et al.*, 1978). Dichloromethane was distilled and passed over basic alumina, prior to use. Difference spectra in visible region were recorded on a Shimadzu 210-A spectrophotometer fitted with a scale expander, using 1 cm pathlength, matched cuvettes. Fresh bilirubin solutions were used in both cuvettes to obtain a baseline in every experiment. Fluorescence spectra were recorded on a Perkin-Elmer MPF-44A spectrofluorimeter.

## Results and discussion

Figure 2 shows the absorption spectrum of bilirubin-IX $\alpha$  in dioxane. An intense band at  $\sim 450$  nm is observed (Lightner, 1982). Addition of 1,2-diaminoethane or 1,2-diaminohexane results in only very small changes in band shape. These effects are clearly illustrated by difference spectroscopy and the results are shown in figure 3 for the case of 1,2-diaminoethane in dioxane. Addition of increasing amounts of the diamine results in an enhancement in intensity at  $\sim 480$  nm and a larger decrease in intensity at  $\sim 450$  nm. At amine:bilirubin ratios  $\gg 2:1$  the features of the difference spectrum change and two positive bands at  $\sim 430$  nm and  $\sim 490$  nm are observed. A plot of changes in optical density versus diamine concentration shows a large discontinuity at an amine:bilirubin ratio of 1. Figure 4 compares the difference spectra for amines of varying chainlength, with the number of spacer methylene groups ( $n$ ) ranging from  $n = 2$  to  $n = 8$ , obtained in  $\text{CH}_2\text{Cl}_2$  solutions at a 1:1 stoichiometry of



**Figure 2.** Absorption spectrum of bilirubin ( $2.65 \times 10^{-5}$  M) in dioxane (----). Absorption spectrum of bilirubin ( $2.65 \times 10^{-5}$  M) - 1,2 diaminoethane ( $3.0 \times 10^{-4}$  M) in dioxane (—).



**Figure 3.** Visible difference spectra of bilirubin ( $2.65 \times 10^{-5}$  M) in the presence of increasing amounts of 1,2 diaminoethane in dioxane. The diamine to bilirubin ratios are, ( $\Delta$ ), 0.2; ( $\times$ ), 0.45; (---), 1.1; (- . -), 1.4; ( $\circ$ ), 1.8; (---), 2.0.

ine and bilirubin. The results of a similar study carried out in dioxane are summarized in figure 5. The difference spectra generated for the diamines with  $n=6$  in this solvent are dramatically different and show a large intensification at

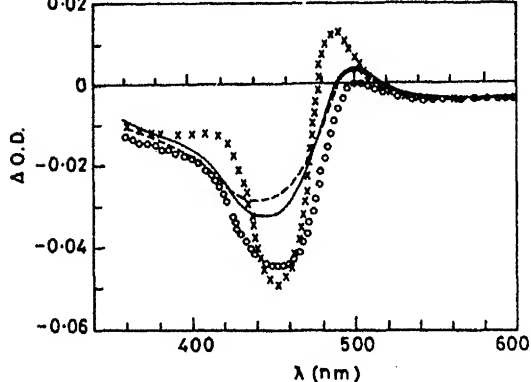


Figure 4. Visible difference spectra in dichloromethane of 1:1 bilirubin-diamine mixtures for the various diamines. Concentration of bilirubin is  $2.19 \times 10^{-5}$  M. (---), 1,2 diaminooethane; (—), 1,4 diaminobutane; (O), 1,6 diaminohexane; (×), 1,8 diaminooctane.

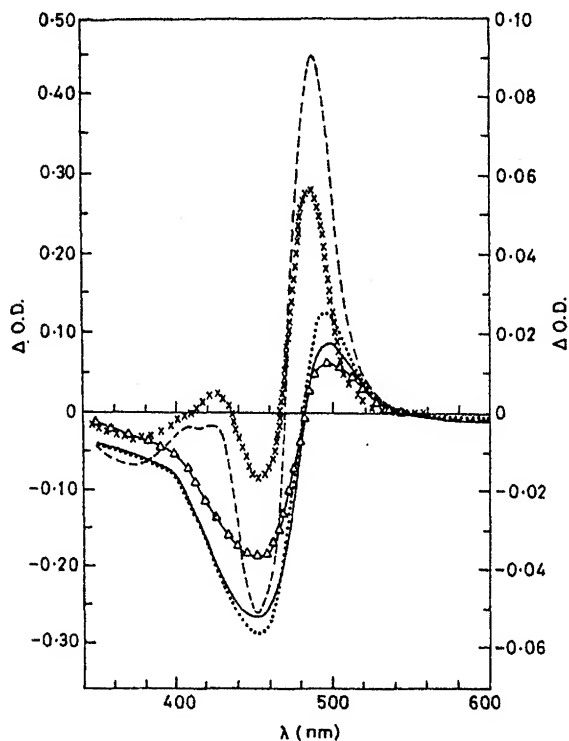


Figure 5. Visible difference spectra in dioxane of 1:1 bilirubin-diamine mixtures for diamines of various chain lengths. Bilirubin concentration is  $2.78 \times 10^{-5}$  M. ( $\Delta$ ), 1,2 diaminooethane; (—), 1,4 diaminobutane; (···), 1,5 diaminopentane; (---), 1,6 diaminohexane; (×), 1,8 diaminooctane. Left hand scale corresponds to the  $n=8$  amine. Note

counts suggest that a significant structural change in the bilirubin chromophore is induced by the altered geometry of the interacting amino groups.

Further confirmation of these observations is obtained from fluorescence spectroscopic studies. Figure 7 shows the effect of addition of 1,2-diaminohexane on the

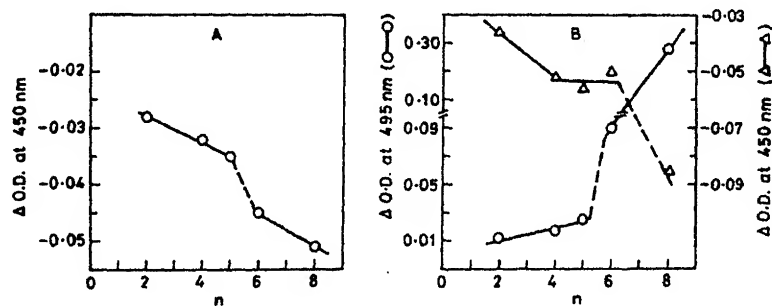


Figure 6. Dependence of the peak and trough intensities of the bilirubin-diamine difference spectra on the methylene chain length (n) of the diamines. (A), Dichloromethane; (B), dioxane.

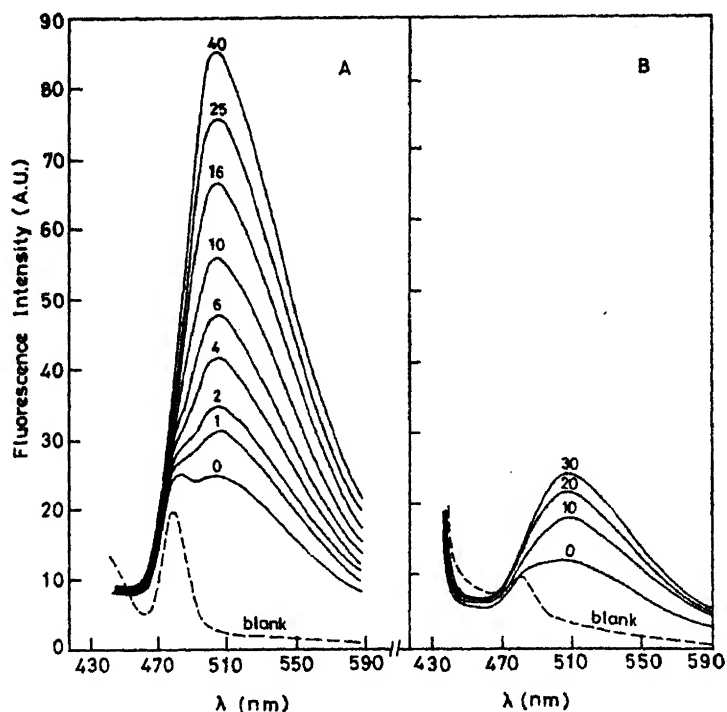
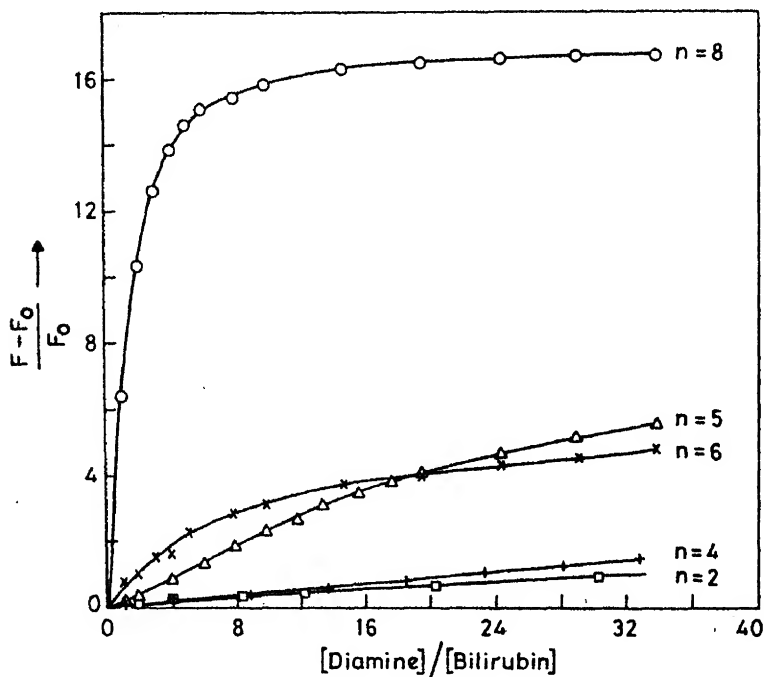


Figure 7. Enhancement of bilirubin ( $6.53 \times 10^{-6}$  M) fluorescence upon the addition of 1,6-diaminohexane in dioxane (A) and dichloromethane (B). The number on each trace indicates the diamine to bilirubin ratio.  $\lambda_{\text{ex}} = 420$  nm.

in both solvents with an emission maximum at  $\sim 515$  nm on exciting at 420 nm (Lightner, 1982; Holzwarth *et al.*, 1980). In dioxane, addition of 1,2-diaminohexane leads to a large enhancement in fluorescence, whereas the intensity increases are much less in dichloromethane solutions. Figure 8 compares the concentration dependence of the fluorescence enhancements observed for various diamines in dioxane. While very small effects are noted for the  $n=2$  to 4 amines, the increases in intensity are larger for  $n=5$  and 6, with the most significant fluorescence enhancement being observed for  $n=8$ . In contrast, in dichloromethane solutions an enhancement of only 2.5 fold in bilirubin emission is observed even in the presence of a 30 fold excess of 1,6-diaminohexane.



**Figure 8.** Enhancement of bilirubin ( $6.53 \times 10^{-6}$  M) fluorescence in dioxane as a function of diamine to bilirubin ratio for various diamines.  $F_0$  is the initial bilirubin fluorescence and  $F$  is the enhanced fluorescence.  $\lambda_{\text{ex}} = 420$  nm,  $\lambda_{\text{em}} = 520$  nm.

The results presented above suggest that in dioxane solutions the conformations of bilirubin in the pigment-amine complex are dependent on the methylene chain length separating the two interacting amino groups. Clearly the nature of the bilirubin species observed for the complexes with  $n=2$  and  $n=8$  are distinctly different. Bilirubin-IX $\alpha$  used in the present study has the 4Z, 15Z configuration about the  $C_4-C_5$  and  $C_{15}-C_{16}$  double bonds (Lightner, 1982). In considering conformational flexibility, torsional freedom about the  $C_5-C_6$ ,  $C_{14}-C_{15}$ ,  $C_9-C_{10}$  and  $C_{10}-C_{11}$  bonds needs to be taken into account. The conformational freedom about the  $C_5-C_6$  and  $C_{14}-C_{15}$  bonds is limited since extensive out of plane distortions can distort

electron delocalization in the pyrrodimethene units (Geddes *et al.*, 1980). The two pyrrodimethene units, however have considerable structural freedom about the C<sub>10</sub>-methylene group, which acts as a flexible hinge (Falk and Muller, 1983). A considerable body of spectroscopic evidence favours an intra-molecularly hydrogen bonded conformation for bilirubin in organic solvents, with two variants illustrated in figure 9. The structures proposed by Knell *et al.* (1972) and Kuenzle *et al.* (1973) vary only in the absence of hydrogen bonding due to the pyrrole NH, in the case of the latter. The crystal structure of bilirubin (Bonnett *et al.*, 1978) established a 'ridge-tile' geometry stabilized by an intramolecular hydrogen bonding pattern similar to that suggested by Knell *et al.* (1972). This conformation is also observed for di-isopropylammonium bilirubinate in the crystalline state (Mugnoli *et al.*, 1978). A major feature of this structure (illustrated in figure 10) is that the two nearly planar dipyrromethene chromophores lie almost perpendicular to one another. The interplanar angles observed in the crystal structure for the two independent molecules are  $\pm 96^\circ$  and  $\pm 99^\circ$  (Bonnett *et al.*, 1978). The O---O distances between the propionate groups calculated from the published coordinates lie between 6.5 and 9.9 Å. The

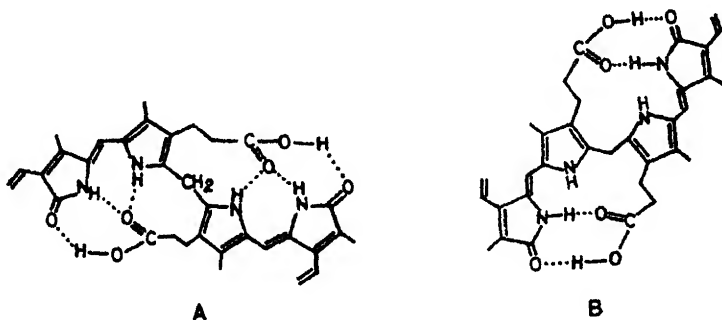


Figure 9. Proposed intramolecularly hydrogen-bonded structures for bilirubin-IX $\alpha$ . Structure A is due to Knell *et al.* (1972) and B is from Kuenzle *et al.* (1973).

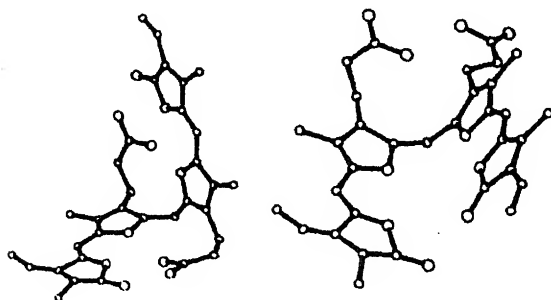


Figure 10. Perspective views of bilirubin conformations (left) 'ridge-tile' structure observed in crystals (Bonnett *et al.*, 1978). Projection plotted using published coordinates (right). 'Quasi-cyclic' bilirubin conformation obtained for values of dihedral angles N<sub>22</sub>-C<sub>9</sub>-C<sub>10</sub>-C<sub>11</sub> ( $\Phi$ )  $\sim -80$  and C<sub>9</sub>-C<sub>10</sub>-C<sub>11</sub>-N<sub>23</sub> ( $\Psi$ )  $\sim 60$ . Note that the conformation rep-

$n=2$  distances calculated for the fully extended (all *trans* poly(methylene chain) diamines, assuming idealized geometries, are  $n=2$  3.8 Å,  $n=4$  6.3 Å,  $n=5$  7.4 Å,  $n=6$  8.8 Å and  $n=8$  11.3 Å. Permitting flexibility about the propionate sidechain, it appears that the  $n=6$  and  $n=8$  diamines can interact comfortably with a 'ridge-tile' bilirubin conformation. On the other hand the  $n=2$  diamine presumably forces bilirubin to adopt a conformation in which the propionate groups are significantly closer together.

We have used molecular modelling studies to generate a stereochemically allowed bilirubin conformation, which is 'quasi-cyclic' or 'porphyrin like' bringing the propionate sidechains into proximity. A steric map corresponding to rotations about the  $C_9-C_{10}$  ( $\Phi$ ) and  $C_{10}-C_{11}$  ( $\Psi$ ) bonds was generated using standard criteria for interatomic contacts (Ramachandran and Sasisekharan, 1968). For the crystal structure geometry the 'virtual dihedral angle'  $C_2-C_8-C_{12}-C_{18}$  ( $\theta$ ) was  $\sim 97^\circ$ , corresponding to a perpendicular arrangement of the dipyrromethene units. A search was made for allowed conformations with low values of  $\theta$  i.e. between  $0^\circ$  and  $30^\circ$ . For conformations with  $\Phi \sim -80^\circ$  and  $\Psi \sim 60^\circ$  a fully allowed structure with  $\theta = -16^\circ$  can be obtained. A perspective view of this conformation is shown in figure 10. One of the propionate sidechain torsion angles has been altered to yield a fully extended sidechain. This bilirubin conformation is favourably disposed for simultaneous interaction of the propionate carboxylate groups with the two amino groups in the shorter diamines. The inter-propionate O---O distances for the idealized conformations in figure 10 vary from 4.5 to 7.6 Å. Flexibility about the propionate C-C bonds can reduce these values further. The above analysis suggests that two distinct families of conformation are possible for bilirubin in dioxane solutions, depending on the nature of the interacting diamine. For  $n \geq 6$  the classical 'ridge-tile' structure is favoured while for  $n \leq 4$  a 'quasi-cyclic' conformation appears suitable. It may be noted that both conformations are chiral and can result in high induced optical activity in complexes with chiral amines (Marr-Leisy *et al.*, 1985).

The fluorescence results suggest that the 'ridge-tile' family of conformations has a high quantum yield while the 'quasi-cyclic' structures have low fluorescence. Dissipation of excitation energy is likely in the latter case by motions involving torsions about the  $C_5-C_6$  and  $C_{14}-C_{15}$  bonds. Quenching of fluorescence due to conformational mobility has indeed been suggested earlier (Lightner, 1982; Holzwarth, 1980). In chloroform and dichloromethane solutions the results suggest that the amines interact with a 'quasi-cyclic' bilirubin conformation, although the evidence is less than compelling. Nevertheless, the results of the present study provide further evidence that both solvent and the nature of the interacting receptor site can serve as conformational determinants for bilirubin.

## Acknowledgements

We are grateful to Dr. Debra Marr-Leisy for carrying out some of the experiments described here and Dr. P. K. C. Paul for help with the molecular modelling studies.

## References

Berthelot, P., Duvaldestin, Ph. and Fevery, J. (1982) in *Bilirubin* (eds K. P. M. Heirwegh and S. B. Brown)

- auer, G. (1983) *Isr. J. Chem.*, **23**, 201.
- auer, G. and Wagniere, G. (1975) *J. Am. Chem. Soc.*, **97**, 1949.
- auer, G., Lavie, E. and Silfen, J. (1977) *Biochim. Biophys. Acta*, **492**, 64.
- onnett, R., Davies, J. E., Hursthouse, M. B. and Sheldrick, G. M. (1978) *Proc. R. Soc. London Ser. B.*, **202**, 249.
- odersen, R. (1980) *CRC Crit. Rev. Clin. Lab. Sci.*, **11**, 305.
- odersen, R. (1982) in *Bilirubin* (eds K. P. M. Heirwegh and S. B. Brown) (Boca Raton, Florida: CRC Press) Vol. 1, p. 75.
- lk, H. and Muller, N. (1983) *Tetrahedron*, **39**, 1875.
- rniss, B. S., Hannaford, A. J., Rugers, V., Smith, P. W. G. and Tatchell, A. R. (1978) *Vogel's Textbook of Practical Organic Chemistry* (London: Longman's) p. 274.
- ddes, A. J., Potterton, E. and Willis, G. (1980) *Photochem. Photobiol.*, **34**, 337.
- olzwarth, A. R., Langer, E., Lehner, H. and Schaffner, K. (1980) *Photochem. Photobiol.*, **32**, 17.
- hell, A. J., Johnson, B. and Hutchinson, D. W. (1972) *Digestion*, **6**, 288.
- enzle, C. C., Weibel, M. H., Pelloni, R. R. and Hemmerich, P. (1973) *Biochem. J.*, **133**, 364.
- ghtner, D. A. (1982) in *Bilirubin* (eds K. P. M. Heirwegh and S. B. Brown) (Boca Raton, Florida: CRC Press) Vol. 1, p. 1.
- ndon, G. L., Park, Y. T. and Lightner, D. L. (1983) *Tetrahedron*, **39**, 1893.
- arr-Leisy, D., Lahiri, K. and Balaram, P. (1985) *Int. J. Peptide Protein Res.*, **25**, 290.
- Donagh, A. F. and Assisi, F. (1972) *Biochem. J.*, **129**, 797.
- ugnoli, A., Manitto, P. and Monti, D. (1978) *Nature (London)*, **273**, 568.
- amachandran, G. N. and Sasisekharan, V. (1968) *Adv. Protein Chem.*, **23**, 283.





# Reactivity of glycoconjugate in membrane system II: can a neutral glycolipid function as lectin receptor in the presence of gangliosides in plasma membrane?

S. K. PODDER\*, A. CHAKRABORTI, K. VIJAYALAKSHMI and P. LALIT KUMAR SINGH

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

**Abstract.** To examine how surface potential controls the reactivity of glycoconjugates at cell surface, the interaction of galactose-specific lectins *e.g.* peanut agglutinin, *Ricinus communis* agglutinin with liposomes bearing asialo GM<sub>1</sub> were studied in the presence of varying amount of ganglioside mixture, GM<sub>n</sub>. The presence of 5% GM<sub>n</sub> causes complete slowing down of precipitin reaction and thereby make carbohydrate moiety of asialo GM<sub>1</sub> completely inaccessible *i.e.* 'cryptic'. In contrast the presence of 1–2% GM<sub>n</sub> enhances the apparent rate and amplitude of the precipitin reaction as surface potential becomes more negative. The relevance of the findings has been discussed in relation to the expression and involvement of the cell-surface sialic acid residues during development and differentiation.

**Keywords.** Lectin receptor; ganglioside; accessibility; distribution in membrane.

## Introduction

In recent years it has been shown from several laboratories that lectin can recognise complex carbohydrate sequence of glycolipids embedded in liposome and thereby give rise to precipitin reaction resembling cell agglutination (Grant and Peters, 1984). The formation of precipitin complex increases the turbidity of the solution in second-minute range and therefore can be studied quantitatively. In the presence of a specific sugar the precipitin complex dissociates (*i.e.* turbidity decreases) with time-constant characteristics of lectin-sugar interaction (Podder *et al.*, 1974). The most interesting feature of the precipitin reaction is that the apparent rate and extent of reaction are controlled by several factors *viz.* (i) surface density of receptors *i.e.* mol per cent of glycolipid content (Surolia *et al.*, 1975), (ii) sequence and length of the carbohydrate chain and an overall lipid composition of the vesicle *i.e.* membrane fluidity, head group interactions etc (Sundler, 1984). The mechanism leading to the required threshold concentration and the drastic enhancement of the initial rate with increase in the ratio of glycolipid to phospholipid is not yet understood. Besides, transmembrane asymmetry, as in the case of GD1a: DPPC system (Thomas and Podder, 1982), size-related topological distribution and surface potential of the vesicle undoubtedly influence the reactivity and accessibility of a given glycoconjugate in a binary system. Whatever be the mechanism it raises the question whether carbohydrate moiety of each glycolipid at the cell surface is available for reaction with lectins and/or carbohydrate specific antibody when a mixture of glycolipids are incorporated in liposome. If not, what controls the accessibility and reactivity of a particular

---

\*To whom all correspondence should be addressed.

Abbreviations used: WGA, Wheat germ agglutinin; PNA, peanut agglutinin; RCA<sub>1</sub>, *Ricinus communis* agglutinin; EPC, egg phosphatidyl choline; NCAM, neuronal cell adhesion molecule; NgCAM, neuronal glial cell adhesion molecule; DML, dimenystayl phosphatidyl choline.

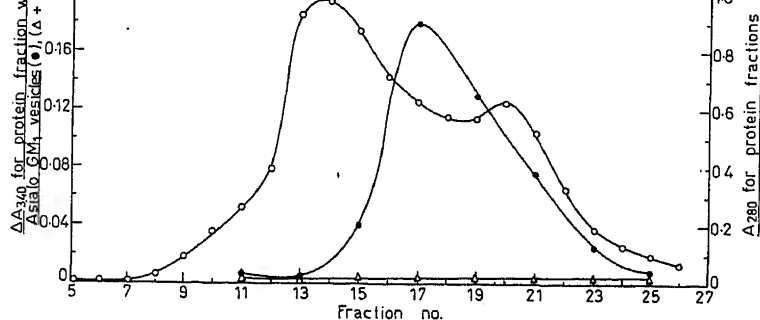
glycolipid in presence of others whose expression at cell surface are often developmentally regulated. This question is of fundamental importance in the assignment of any marker glycoconjugate to have an *in vivo* receptor function and act as antigenic determinant during development, differentiation and oncogenic transformation. For better understanding of the molecular basis of individual factors (e.g. fluidity, surface density, surface potential) that influence the kinetics and specificity, attempts have been made to develop suitable liposomal system for lectins like wheat germ agglutinin (WGA), peanut agglutinin (PNA), *Ricinus communis* agglutinin (RCA<sub>1</sub>).

Recently we have investigated the formation of precipitin complexes between vesicles containing 5 mol per cent of asialo GM<sub>1</sub> and galactose specific lectins like ricin, RCA<sub>1</sub> and PNA. The rate and extent of lactose-induced dissociation are found to be very sensitive to the concentration of lactose and the type of lectin. The data obtained from quantitative analysis of sugar-induced dissociation rate of precipitin complex suggest that specificity of interaction between lectin and complex carbohydrate moiety of glycosphingolipid is at least two order magnitude higher than that of simple sugar. In the case of PNA it is even higher and comparable to high affinity binding site at the cell surface (Singh *et al.*, 1986). Moreover the kinetics of formation and dissociation of precipitin complex is slower. In contrast the dissociation rate in case of ricin and RCA<sub>1</sub> is faster and only 36% of the total complex can be monitored under experimental set up. Because of its reported mitogenicity, kinetic property and uses in the study of lymphocyte maturation (Reisner *et al.*, 1976), PNA-asialo GM<sub>1</sub> system is chosen for further study to examine the influence of other glycolipids on the reactivity of asialo GM<sub>1</sub> towards PNA. Hence we wish to report how ganglioside when incorporated in the same vesicle containing asialo GM<sub>1</sub> affect its reactivity towards galactose specific lectins. Data presented here show that at about 5 mol per cent of mixed gangliosides, asialo GM<sub>1</sub> is inaccessible to PNA and RCA<sub>1</sub>. In contrast, in the presence of lesser mol per cent of GM<sub>m</sub>, accessibility increases.

## Materials and methods

Egg yolk lecithin was isolated using the method of Litman (1973) and total mixed ganglioside by the procedure of Folch *et al.* (1957). Asialo GM<sub>1</sub> was prepared from mixed ganglioside by acid hydrolysis with 0.1 N H<sub>2</sub>SO<sub>4</sub> at 80°C for 1 h. The purity of the gangliosides were checked by thin-layer chromatography methods using resorcinol and orcinol reagents for mixed gangliosides and asialo GM<sub>1</sub>, respectively. RCA<sub>1</sub> was isolated from the locally available seeds using DEAE-Sephadex followed by G-100 column chromatography (Douglas *et al.*, 1978). The activity was detected by guar gum and asialo GM<sub>1</sub> vesicles by measuring the increase in turbidity at 340 nm.

PNA was isolated from local seeds using Sepharose 6B affinity chromatography (Terao *et al.*, 1975). Figure 1 shows the elution profile of 60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitated proteins from peanut seeds. PNA was detected by liposomal assay using vesicles containing 5 mol per cent asialo GM<sub>1</sub> in the absence and presence of galactose. Agglutination reaction of mouse thymocytes gives the hemagglutinating activity of PNA as 2000 HU/mg of protein approximately. The assay thus described, avoids the complicated procedure of treating the erythrocytes with neuraminidase for activity assay of PNA. Purity of both the proteins were checked by sodium

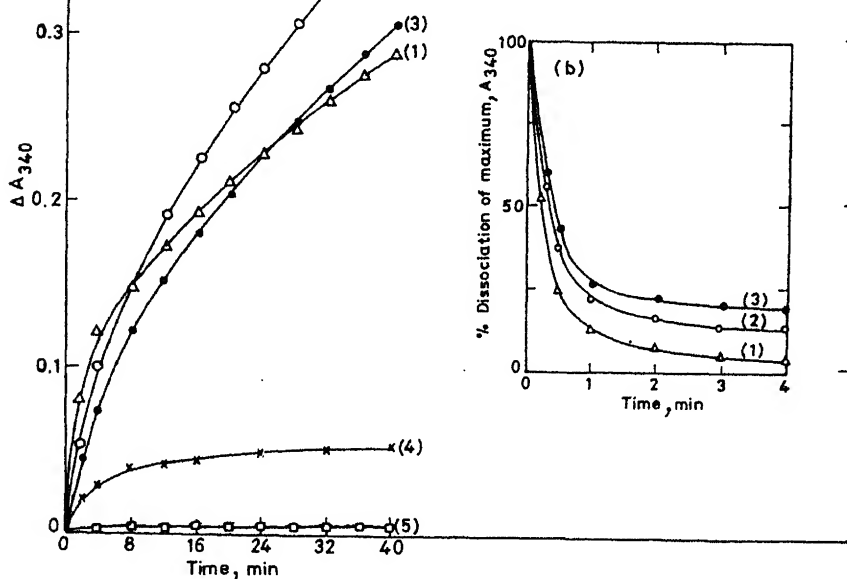


**Figure 1.** Elution profile of 60%  $(\text{NH}_4)_2\text{SO}_4$  fraction of peanut seeds (*Arachis hypogaea*) on Sepharose 6B column ( $2 \times 45$  cm). Protein loaded 30'31 mg, fraction volume 7 ml, flow rate 7 ml/h.

Egg phosphatidyl choline (EPC) vesicles containing various types of gangliosides are prepared by sonication for 20 min till it becomes visually clear. Its size and stability were characterized by measuring the absorbance as well as the initial velocity of precipitin reaction with lectins.

## Results

Although co-solubilisation of lecithin and mixture of glycolipid would lead to the formation of liposomes having carbohydrate moiety of glycoconjugate exposed at outer surface during sonication, the reactivity of exposed carbohydrate moiety towards lectins may change with time due to the vesicle instability resulting from the formation of either large unilamellar or multilamellar vesicle upon fusion of aggregated small vesicles. It could also result from the slow transmembrane flip-flop of incorporated glycoconjugate. To circumvent the problem, sonicated vesicles were annealed for a longer period of time (24-48 h) before use. During this course it was observed that long period of annealing was necessary to obtain precipitin reaction of constant amplitude. It may be mentioned that the annealing time is sensitive to duration of sonication, nature of lecithin as well as mol per cent of glycoconjugate. It also depends on the time history of annealing and storage condition. The details of these findings will be published elsewhere. Figure 2 shows the precipitin reaction of PNA with liposome containing 5 mol per cent asialo  $\text{GM}_1$  in the presence of various amounts of mixed gangliosides in the same liposome. It is clear that the addition of  $\text{GM}_n$  though reduced initial reaction of precipitin reaction presumably due to electrostatic repulsion, the amplitude at 40 min for vesicles containing 1% and 2%  $\text{GM}_n$  is about 1.3 and 1.1 times increases respectively, than that of asialo  $\text{GM}_1$  vesicle alone. From the absorption/mM  $\text{PPO}_4$  value shown in table it is seen that the size of the lecithin vesicle is dependent on the amount of  $\text{GM}_n$  content in the vesicle. To what extent the observed difference in amplitude of precipitin reaction can be related to the difference in size and topological distribution



**Figure 2.** Kinetics of precipitin reaction of liposomes bearing asialo GM<sub>1</sub> (5 mol per cent) with various amounts of gangliosides with PNA (a) and its dissociation (b) in the presence of 23.33 mM galactose.

Per cent mol content of GM<sub>n</sub>: (1) without GM<sub>n</sub>; (2) 1; (3) 2; (4) 3; (5) 2 per cent GM<sub>n</sub> alone; also 5 per cent GM<sub>n</sub>.

need to be ascertained. In figure 3, the effect of salt on the rate of precipitin reaction is shown. From such kinetic data, the interaction specificity of carbohydrate lectin interaction was calculated as described earlier (Singh *et al.*, 1986). These are summarised in table 1 and table 2. Some of the preliminary data obtained for free RCA<sub>1</sub> and liposome bound RCA<sub>1</sub> are also included in the table 1. In the case of liposome bound RCA<sub>1</sub> the presence of GM<sub>n</sub> in the same liposome reduced the affinity constant  $K_m$  by about 10 times while that of free RCA<sub>1</sub> reduced marginally. This is not observed in the case of PNA where the affinity value remains almost same. It is not known whether this difference could be ascribed to the difference in pI values of PNA (5.96) and RCA<sub>1</sub> (7.1). It may be emphasised here that the calculated value is found to be insensitive to the observed variation of the amplitude of precipitin reaction which is critically dependent on the time history of the sample as well as nature and composition of the vesicles.

## Discussion

From the results presented here it appears that ganglioside GM<sub>n</sub> embedded in the liposome containing asialo GM<sub>1</sub> can modulate the rate and extent of precipitin

**Table 1.** Characteristics of precipitin reaction of liposomes containing various types of gangliosides with galactose specific lectins.

Lectin	Type of vesicle	A/mMPO <sub>4</sub> of vesicle at 560 nm x 10 <sup>2</sup>	conc. of asialo GM <sub>1</sub> (μM)	v <sub>i</sub> (% of amplitude at 1 min/30 min)	Dissociation with 23.33 mM Gal. (%)	t <sub>1/2</sub> (s)	k <sub>d</sub> (s <sup>-1</sup> )	K** (M <sup>-1</sup> )
<b>*EPC vesicle with 5 mol per cent of asialo GM<sub>1</sub></b>								
PNA 2.5 μM	without GM <sub>n</sub>	1.29	20	9.7	96.5	14.4	2.18 × 10 <sup>-3</sup>	1.7 × 10 <sup>7</sup>
	1% GM <sub>n</sub>	1.27	20	9.35	87.86	19.2	1.64 × 10 <sup>-3</sup>	2.25 × 10 <sup>7</sup>
	2% GM <sub>n</sub>	1.38	20	6.6	88.26	19.2	1.64 × 10 <sup>-3</sup>	2.25 × 10 <sup>7</sup>
	3% GM <sub>n</sub>	0.9	20	2.5	92.33	19.2	1.64 × 10 <sup>-3</sup>	2.25 × 10 <sup>7</sup>
<b>†EPC vesicle with 5 mol per cent of asialo GM<sub>1</sub></b>								
RCA† 2.2 μM	without GM <sub>n</sub>	—	20	no reaction	—	—	—	—
	1% GM <sub>1</sub>	2.69	20	21.4	53.53	12.7	0.37	1.2 × 10 <sup>6</sup>
	2% GM <sub>n</sub>	1.8	20	16.0	71.19	7.26	0.65	0.7 × 10 <sup>6</sup>
	3% GM <sub>n</sub>	1.52	20	15.79	80.50	7.26	0.65	0.7 × 10 <sup>6</sup>
	2% GM <sub>n</sub> alone	1.03	20	very slow reaction no reaction	—	—	—	—
RCA <sub>1</sub> 2.1 μM	5% asialo GM <sub>1</sub> in DML vesicle	0.124	12.4	71	(11.5 mM) 97.3	24	2.0 × 10 <sup>-3</sup>	2.2 × 10 <sup>8</sup>
	2% GM <sub>n</sub> + 5% asialo GM <sub>1</sub> in DML vesicle	0.155	20	38	(2.4 mM) 93	17	1.4 × 10 <sup>-2</sup>	3.2 × 10 <sup>7</sup>

\*Vesicle used after 3 days of storage.

†On the 5th day.

\*\*Conjugated RCA<sub>1</sub> in DML vesicle.

\*\*K<sub>d</sub> calculated using K<sub>f</sub> for lectin-galactose system.

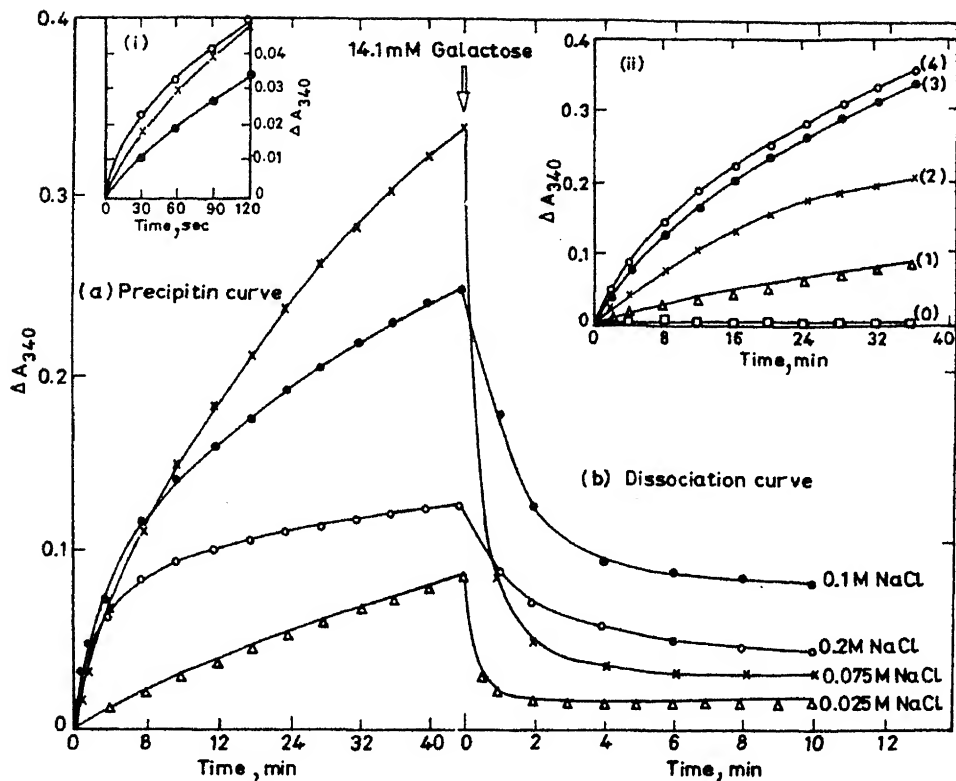


Figure 3. Precipitin reaction of vesicles containing 5 mol per cent of asialo GM<sub>1</sub> and 2 mol per cent of GM<sub>2</sub> with PNA with varying amounts of salt (NaCl) and their subsequent dissociation in the presence of 14.1 mM galactose. Concentration of PNA (2.5  $\mu$ M) and of asialo GM<sub>1</sub> (20  $\mu$ M).

Inset (i). Expanded scale of initial rate of precipitin reaction.

Inset (ii). Precipitin reaction of vesicles, with different concentration of NaCl. (0) without salt; (1) 25 mM; (2) 50 mM; (3) 65 mM; (4) 75 mM.

reaction even when present to the extent 1–3 mol per cent of total lipid. At 5 mol per cent of GM<sub>n</sub>. The complete slowing down of rate of the precipitin reaction was observed. Similar modulation by ganglioside have been reported by Alving *et al.* (1980), in the case of binding of antibodies to liposomal phospholipids as well as neutral glycolipids *viz.* globosides, galactocerebrosides, Forsman antigens etc. Endo *et al.* (1982) reported the inhibition of antigenic activity of liposomal Forsman antigen by glycophorin which contains several saccharides terminated by NANA and bind to WGA. The observed inhibition by sialylglycoconjugates is attributed to either carbohydrate-carbohydrate interaction in glycoconjugates and/or head group interaction between carbohydrate moiety of ganglioside and phospholipids.

It is known that even though sialic acid residues of embedded ganglioside are at a significant distance from the surface, the electrostatic potential with lecithin-ganglioside liposome becomes more negative as the mol content of GM<sub>n</sub> increases. With

Table 2. Effect of salt on the precipitin reaction of liposomes bearing different gangliosides with PNA.

Lectin	Characteristics of vesicle	Salt conc. (mM NaCl)	$v_i$ % of amplitude at 2 min/40 min	Dissociation with 14.11 mM gal. (%)	$t_d$ (s)	$k_d$ ( $s^{-1}$ )	$K_a$ ( $M^{-1}$ )
PNA (2.5 $\mu M$ )	1. EPC vesicle with 5 mol per cent asialo GM <sub>1</sub> and 2 mol per cent GM <sub>2</sub>	0	no reaction	—	—	—	—
		25	8.7	90	16.8	$3 \times 10^{-3}$	$1.2 \times 10^7$
		35	7.14	97	21.8	$2.4 \times 10^{-3}$	$1.5 \times 10^7$
	2. A/mMPO <sub>4</sub> at 560 nm = $1.38 \times 10^{-2}$	50	8.98	—	—	—	—
	3. Conc. of asialo GM <sub>1</sub> (20 $\mu M$ )	65	10.64	—	—	—	—
		75	12.25	94.8	33.6	$1.54 \times 10^{-3}$	$2.4 \times 10^7$
		100	17.5	64.9	48.48	$1.07 \times 10^{-3}$	$3.4 \times 10^7$
		200	31.4	62.5	67.2	$0.77 \times 10^{-3}$	$4.8 \times 10^7$



surface potential and topological distribution of embedded glycoconjugates are far more important in controlling the rate and extent of the precipitin reaction. This view can be further tested by measuring the rate of precipitin reaction when other negatively charged phospholipids *i.e.* phosphatidyl glycerol and phosphatidyl serine are present in the liposome instead of GM<sub>1</sub>. Whatever be the mechanism of the observed modulation of the reaction between lectin and carbohydrate moiety of asialo GM<sub>1</sub> the data presented here allows us to draw the following conclusion. The individual ganglioside though function as specific receptor will always influence other receptor-ligand interactions at the cell surface by modulating surface charge density and head group interaction resulting in the changed conformation of exposed carbohydrate moiety and its topological distribution. At higher content of charged gangliosides it can completely slow down the rate of reaction as reported here and thereby make the exposed carbohydrate at the outer surface completely inaccessible. In other words, exposed carbohydrate would appear cryptic. This secondary influence of sialic acid residue on the accessibility of other surface carbohydrates is distinct from that resulting from masking due to incorporation of sialic acid residue at the terminal galactose residue by sialyltransferase action (Toporowicz and Reisner, 1986). Thus, there need not be any direct relation between changes in sialic acid content and expression of functional receptors in differentiation and other carbohydrate mediated phenomena particularly those are developmentally regulated *via.* adhesion molecules like NCAM and NgCAM (Edelman, 1985).

## Acknowledgements

This work is financially supported by Council of Scientific and Industrial Research and Department of Science and Technology, New Delhi.

## References

- Alving, C. R., Urban, K. A. and Richards, R. L. (1980) *Biochim. Biophys. Acta*, **600**, 117.
- Douglas A. Lappi, Wolfgang Kapmeyer, Janice, M., Beglau and Nathan O. Kaplan (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1096.
- Edelman, M. Gerald (1985) *Annu. Rev. Biochem.*, **54**, 135.
- Endo, T., Nojima, S. and Inoue, K. (1982) *J. Biochem.*, **92**, 1883.
- Folch, J., Lees, M. B. and Sloane Stanley, G. H. (1957) *J. Biol. Chem.*, **226**, 497.
- Grant, C. W. M. and Peters, M. W. (1984) *Biochim. Biophys. Acta*, **779**, 403.
- Laemmli (1970) *Nature (London)*, **227**, 680.
- Litman, B. J. (1973) *Biochemistry*, **12**, 2545.
- Singh Lalit Kumar P., Vijayalakshmi, K. and Podder, S. K. (1986) *J. Indian Chem. Soc.*, **60**, 123.
- Podder, S. K., Surolia, A. and Bachhawati, B. K. (1974) *Eur. J. Biochem.*, **44**, 151.
- Reisner, Y., Linker Isreali, M. and Sharon, N. (1976) *Cell. Immunol.*, **25**, 129.
- Sundler, R. (1984) *Biochim. Biophys. Acta*, **771**, 59.
- Surolia, A., Bachhawati, B. K. and Podder, S. K. (1975) *Nature (London)*, **257**, 802.
- Terao, T., Irimura, T. and Osawa, T. (1975) *Hoppe-Seyler's Z. Physiol. Chem.*, **356**, 1685.
- Thomas, P. D. and Podder, S. K. (1982) *Biochim. Biophys. Acta*, **688**, 453.
- Toporowicz, A. and Reisner, R. (1986) *Cell. Immunol.*, **100**, 10.

## Mechanism of protein synthesis inhibition by vaccinia viral core and reversal of this inhibition by reticulocyte peptide chain initiation factors

DEBOPAM CHAKRABARTI, KOLLURU V. A. RAMAIAH,  
ANANDA L. ROY, MILAN BAGCHI and NABA K. GUPTA\*

Department of Chemistry, The University of Nebraska, Lincoln, Nebraska U.S.A.  
68588-0304

**Abstract.** Vaccinia viral core inhibits protein synthesis in heme-supplemented reticulocyte lysate. A reticulocyte cell supernatant factor, which reversed protein synthesis inhibition in heme-deficient reticulocyte lysate also reversed vaccinia viral core induced protein synthesis inhibition in heme-supplemented reticulocyte lysate. Significant inhibition reversal activity was also observed with a partially purified eukaryotic initiation factor-2 preparation and this activity was lost upon further purification of eukaryotic initiation factor-2. The ribosomal salt-wash factor Co-eukaryotic initiation factor-2 which like reticulocyte supernatant factor contains guanine nucleotide exchange factor activity, was completely inactive. Vaccinia viral core induced detectable level of eukaryotic initiation factor-2  $\alpha$ -subunit phosphorylation when incubated in the heme-supplemented reticulocyte lysate. This lysate preparation contains guanine nucleotide exchange factor activity. However, when the same reticulocyte lysate was previously incubated with the vaccinia viral core, the guanine nucleotide exchange factor activity during subsequent incubation was almost completely inhibited.

**Keywords.** Vaccinia virus; protein synthesis; peptide chain initiation factor.

## Introduction

The infection of animal cells with viruses is often accompanied by shut-off of host protein synthesis. There are indications that some step(s) in peptide chain initiation is involved in this inhibition (Bablanian, 1975). Recently several laboratories have reported that protein synthesis inhibition in cell-free extracts obtained from virus-infected cells could be reversed by the addition of exogenous peptide chain initiation factors such as eukaryotic initiation factor-2 (eIF-2) (Centralla and Lucas-Lennard, 1982; Dratewka-Kos *et al.*, 1984; Reichel *et al.*, 1985; Siekerka *et al.*, 1985) guanine nucleotide exchange factor (GEF) (Dratewka-Kos *et al.*, 1984; Reichel *et al.*, 1985; Siekerka *et al.*, 1985) eIF-4B (Dratewka-Kos *et al.*, 1984; Van Steeg *et al.*, 1984) and eIF-4F (Centralla and Lucas-Lennard, 1982; Griffo *et al.*, 1985; Edery *et al.*, 1983) indicating possible alterations of these factor(s) during virus infection.

In the case of vaccinia virus, protein synthesis inhibition occurs in the absence of viral RNA or new protein synthesis (Shatkin, 1965; Moss, 1968; Rosemond-Horntseak

to the first stage of uncoating i.e. the release of cores into the cytoplasm (Joklik, 1969; Dalls, 1965). Using a cell-free system, Ben Hamida and Beaud (1978) have reported that vaccinia viral core inhibits protein synthesis and also Met-tRNA<sub>f</sub>-40S initiation complex formation in reticulocyte lysate (Parson *et al.*, 1980). Recently, Coppola and Bablanian (1983) have reported that the vaccinia virus transcripts generated *in vitro* by transcription of viral core, inhibited translation of cellular and encephalomyocarditis virus (EMC) mRNA but not that of vaccinia viral mRNA in reticulocyte lysate.

Several years ago, we reported (Ghosh-Dastidar *et al.*, 1981) that vaccinia viral core inhibits Met-tRNA<sub>f</sub>-40S initiation complex formation in reticulocyte lysate in response to physiological mRNAs and not in response to AUG codon indicating that viral core inhibits some step(s) in peptide chain initiation involved in recognition of the structural features unique to physiological mRNAs. In this paper, we report that a reticulocyte cell supernatant factor, (RF) and a partially purified eIF-2 preparation which reverse protein synthesis inhibition in heme-deficient reticulocyte lysate (Gross, 1976; Ranu and London, 1977; Amsez *et al.*, 1979; Ralston *et al.*, 1979; Grace *et al.*, 1982, 1984; Siekierka *et al.*, 1981, 1982, 1983; Matts *et al.*, 1983; Konieczny and Safer, 1983) also reverse vaccinia viral core induced protein synthesis inhibition in reticulocyte lysate. The possible mechanism of vaccinia viral core induced protein synthesis inhibition and its reversal by RF have been discussed.

A preliminary report of this work has been presented (Chakrabarti *et al.*, 1985).

## Materials and methods

The sources of most of the materials used in these studies were the same as described previously (Ghosh-Dastidar *et al.*, 1980, 1981; Ralston *et al.*, 1979; Grace *et al.*, 1982, 1984; Majumdar *et al.*, 1979; Das *et al.*, 1982; Chakravarty *et al.*, 1985; Bagchi *et al.*, 1984). [<sup>35</sup>S]-Methionine (1490 Ci/mmol) was obtained from Amersham/Searle. [<sup>3</sup>H]-GDP (13 Ci/mmol) and [<sup>14</sup>C]-Leucine (344 mCi/mmol) were obtained from New England and Nuclear and [<sup>γ</sup><sup>32</sup>P]-ATP (4500 Ci/mmol) was purchased from ICN.

### *Reticulocyte ribosomes, ribosomal subunits and peptide chain initiation factors*

Reticulocyte ribosomes, 40S ribosomal subunits and ribosomal salt (0.5 M KCl) wash were prepared as described previously (Chakravarty *et al.*, 1985). Partially purified eIF-2 (Fraction III) and Co-eIF-2 preparations were obtained after a CM-Sephadex chromatography step, as described previously (Das *et al.*, 1982). The eIF-2 activity was further purified using a hydroxylapatite column chromatographic procedure (Fraction IV). The hydroxylapatite column was equilibrated with Buffer A containing 10 mM potassium phosphate, 10% glycerol, 5 mM β-mercaptoethanol and 50 μM EDTA. The Fraction III eIF-2 preparation was applied onto the column. The column was washed with 3 column volumes of Buffer A containing 200 mM potassium phosphate (pH 7.8) and the proteins were then eluted from the column using a potassium phosphate (pH 7.8) gradient (200 mM → 500 mM) in Buffer A. The

eIF-2 activity eluted at potassium phosphate concentration range around 300 mM. The fractions showing peak eIF-2 activity were pooled, and the pooled fraction was dialyzed against a buffer containing 20 mM Tris-HCl, 10% glycerol, 5 mM  $\beta$ -mercaptoethanol and 50  $\mu$ M EDTA. The dialyzed solution was concentrated by Centricon micro concentrator and stored in small aliquots in liquid nitrogen (Fraction IV).

The Co-eIF-2 activity after CM-Sephadex chromatography was further purified using DEAE-cellulose chromatography (Das *et al.*, 1982). The reticulocyte cell supernatant factor RF was purified following the procedure of Grace *et al.* (1984). As before, Fraction V RF preparation contained significant amounts of eIF-2 activity and the bulk of this eIF-2 activity was removed at the next purification step using CM-Sephadex column chromatography (Fraction VI).

#### *Preparation of viral cores*

Vaccinia virus was isolated and purified according to the method of Joklik (1962) and Levins and Joklik (1977). Viral core was isolated and purified as described before Ghosh-Dastidar *et al.*, 1981). Purified cores were resuspended by sonication in 10 mM Tris-HCl, pH 8.4.

#### *Preparation of rabbit reticulocyte lysates and assay of protein synthesis*

The procedures for the preparation of reticulocytes and reticulocyte lysates have been described (Ghosh-Dastidar *et al.*, 1980, 1981). Protein synthesis was assayed by the incorporation of [ $^{14}$ C]-Leucine into hot trichloroacetic acid-insoluble protein.

#### *Peptide chain initiation assays*

eIF-2 and GEF activity in different factor preparations were assayed using the standard millipore filtration assay method as described previously (Chakravarty *et al.*, 1985; Bagchi *et al.*, 1984).

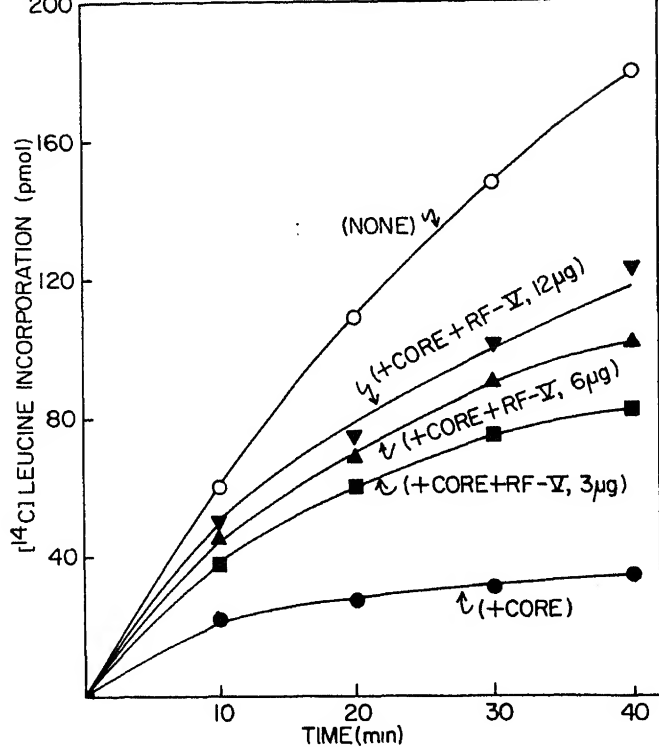
Details of the individual experimental procedures have also been described.

#### *Gel Electrophoresis*

SaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) using a 10% acrylamide/0.16% N,N'-methylenebisacrylamide gel. Autoradiograms were made with Kodak X-OMAT AR X-ray film.

## **Results**

We studied the effects of addition of vaccinia viral core and the reticulocyte cell



**Figure 1.** Effects of addition of vaccinia viral core and the reticulocyte cell supernatant factor (RF, Fraction V) on protein synthesis in reticulocyte lysate.

The reaction mixture containing 15  $\mu$ l reticulocyte lysate, 75 mM KCl, 37  $\mu$ M Hemin-Cl was preincubated at 25°C for 10 min with 0.11  $A_{260}$  unit of vaccinia viral core as indicated.

The reaction mixture (final volume 45  $\mu$ l) was then mixed with 37.5  $\mu$ M amino acid mixture (-Leu), 38  $\mu$ M [ $^{14}$ C]-Leucine incorporation in 20  $\mu$ l aliquot was determined by standard method.

after further addition of protein synthesis components and RF as indicated. The reticulocyte lysate efficiently incorporated [ $^{14}$ C]-Leucine and the addition of excess RF marginally stimulated such activity. However, preincubation with the viral core, reduced the protein synthesis activity of the lysate to approximately 20%. Addition of RF significantly reversed this viral core induced protein synthesis inhibition and this reversal activity increased with increasing RF concentration. Approximately, 70% of the original protein synthesis activity of the lysate was restored in the presence of 12  $\mu$ g RF.

We compared the activities of several factor preparations, such as RF (Fraction V and VI) Co-eIF-2 and eIF-2, for reversal of viral core induced protein synthesis inhibition in reticulocyte lysate (table 1). RF V fraction contained significant amounts of both eIF-2 and GEF activities and bulk of the eIF-2 activity was removed by further purification using CM-Sephadex chromatography (RF Fraction VI). Fraction VI RF preparation was enriched in GEF activity. The ribosomal salt wash factor Co-eIF-2 does not reverse protein synthesis inhibition in heme-deficient

**Table 1.** Activities of different factor preparations for reversal of vaccinia viral core induced protein synthesis inhibition in reticulocyte lysate.

Factor(s) Added	Amount ( $\mu$ g)	[ $^{14}$ C]-Leu incorporated pmol/20 $\mu$ l incubation mixture	Activity (%)
None	—	240	100
Viral core	—	60	25
Viral core + RF V	4	140	58
Viral core + RF VI	4	100	42
Viral core + Co-eIF-2	40	70	29
Viral core + eIF-2 III	4	120	50
Viral core + eIF-2 IV	8	70	29

Heme-supplemented reticulocyte lysate was incubated with 0.11 A<sub>260</sub> unit vaccinia viral core for 10 min at 25°C. Amino acid incorporation into protein was then determined using standard assay conditions.

(table 1), both Fraction V and VI RF preparations reversed viral core induced protein synthesis inhibition although ternary complex activity of Fraction VI RF preparation was significantly less. The Co-eIF-2 preparation even at 40  $\mu$ g concentration did not have any significant effect on protein synthesis. A partially purified eIF-2 preparation (Fraction III approximately 50% pure as judged by sodium dodecyl sulphate-gel) showed significant protein synthesis inhibition reversal activity. However, upon further purification using a hydroxylapatite chromatography (Fraction IV), this protein synthesis inhibition reversal activity was lost. The purity of the Fraction IV eIF-2 preparation is over 80% and this fraction shows mainly 3 polypeptide bands characteristic of eIF-2.

To determine the component activity or activities in RF and eIF-2 preparations responsible for reversal of viral core induced protein synthesis inhibition, we analyzed the GEF and eIF-2 activities in these preparations (tables 2 and 3). As shown in table 2, the GEF activity in both Fraction V and VI RF preparation was comparable whereas the GEF activity in Co-eIF-2 preparation was significantly less (approximately 10% of that observed with Fraction V and VI RF preparations). The eIF-2 preparations (Fraction III and IV) showed no detectable level of GEF activity (data not shown here). It should be emphasized that the experiments described in table 2 were performed using RF V, VI and Co-eIF-2 preparations containing comparable amounts of GEF activity. As shown in tables 1 and 2, there is no direct correlation between GEF activity and protein synthesis inhibition reversal activity.

The eIF-2 activities in different factor preparations are shown in table 3. The eIF-2 activities were assayed in the absence of Mg<sup>2+</sup> and in the presence of excess Co-eIF-2 (Grace *et al.*, 1984). Co-eIF-2 preparations were completely devoid of eIF-2 activity. As shown here Fraction V RF preparation contains some eIF-2 activity and Fraction VI RF preparation is almost devoid of eIF-2 activity. The partially purified eIF-2 preparation (eIF-2 III) was fully active in ternary complex formation. Almost 100%

**Table 2.** Comparison of GEF activity in different factor preparations.

Factor	Amount ( $\mu\text{g}$ )	$[\text{}^3\text{H}]$ -GDP displaced (%)
RF-V	1.0	46
	2.0	58
RF-VI	1.0	50
	2.0	63
Co-eIF-2	10.0	41
	20.0	59

eIF-2- $[\text{}^3\text{H}]$ -GDP was preformed (5 min,  $37^\circ$ ) in a reaction mixture (total vol.,  $50\ \mu\text{l}$ ) containing 25 mM Tris-HCl (pH 7.8), 90 mM KCl, 2.5 mM dithiothreitol, bovine serum albumin, 160  $\mu\text{g}/\text{ml}$ ; eIF-2 1.5  $\mu\text{g}$  and 1.5  $\mu\text{M}$   $[\text{}^3\text{H}]$ -GDP (5400 cpm/pmol). After the incubation  $\text{Mg}(\text{OAc})_2$  and unlabelled GDP was added to a final concentration of 1 mM and 100  $\mu\text{M}$  respectively. eIF-2- $[\text{}^3\text{H}]$ -GDP ( $50\ \mu\text{l}$ ) and different amount of factor preparation was incubated at  $37^\circ\text{C}$  for 15 seconds. Ice-cold wash buffer (3 ml) containing 20 mM Tris-HCl (pH 7.8), 100 mM KCl and 1 mM  $\text{Mg}(\text{OAc})_2$  was added to stop the reaction. The reaction mixtures were then assayed using standard Millipore filtration conditions for  $[\text{}^3\text{H}]$ -GDP release.

**Table 3.** Ternary complex formation by different factor preparations.

Factor	$[\text{}^{35}\text{S}]$ Met-tRNA <sub>f</sub> bound (pmol)
RF V	1.0
RF VI	0.1
eIF-2 III	5.0
eIF-2 IV	2.3

Standard Millipore filtration assay conditions were used in the presence of 15  $\mu\text{g}$  Co-eIF-2. Amounts of different factors used were 1.5  $\mu\text{g}$  each.

freed of contaminating proteins. However, as reported earlier, extensively purified eIF-2 preparations loses activity rapidly upon storage, and as shown in table 3, approximately 25–30% of the potentially active eIF-2 molecules formed ternary complexes. As reported in table 1, RF V, VI and eIF-2 preparations reversed viral capsid induced protein synthesis inhibition. The results presented in table 3, clearly

inhibition. Clearly, this reversal activity in eIF-2 preparation is not related to GEF activity. This activity was completely lost upon further purification of eIF-2 using hydroxylapatite chromatography (Fraction IV). Fraction IV eIF-2 is fully active in several partial reactions studied including ternary and Met-tRNA<sub>f</sub>40S complex formation. The characteristics of the protein synthesis inhibition reversal activity in eIF-2 III preparation is not clear. Apparently, the presence of eIF-2 activity cannot explain the protein synthesis inhibition reversal activities in RF Fractions V and VI. As shown in table 3, RF Fraction VI is almost completely devoid of eIF-2 activity.

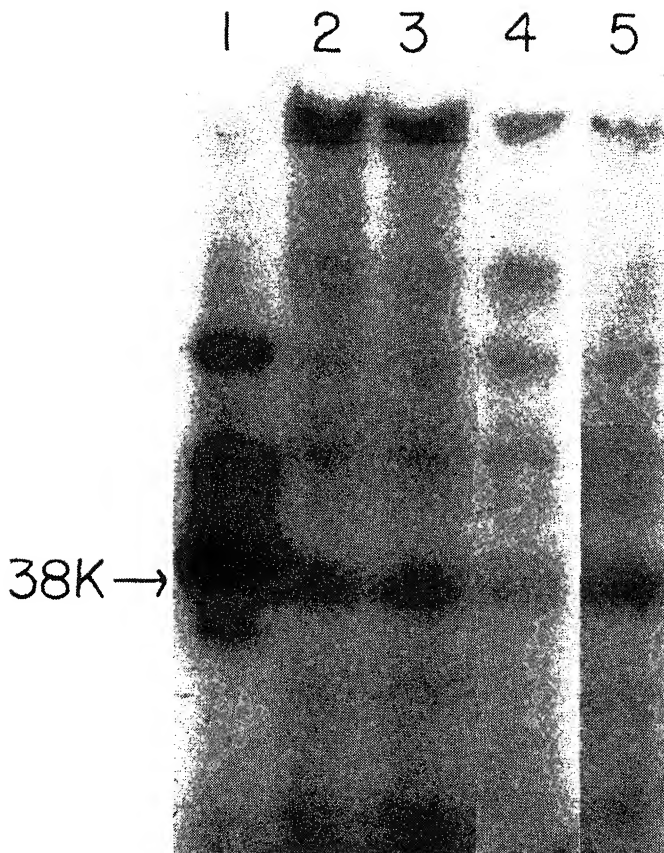
Because of the involvement of RF in protein synthesis inhibition reversal, we considered the possibility of eIF-2  $\alpha$ -subunit phosphorylation during this inhibition. The eIF-2  $\alpha$ -subunit phosphorylation was compared in both heme-deficient reticulocyte lysate and also in heme-supplemented lysate in the presence of viral core (figure 2). Exogenous eIF-2 was used in these experiments. In both cases, there was significant increase in eIF-2  $\alpha$ -subunit phosphorylation over control experiments; heme-deficient lysate (figure 2, lane 3) *vs* heme-supplemented lysate (figure 2, lane 2) and heme-supplemented lysate with viral core (figure 2, lane 5) *vs* heme-supplemented lysate without viral core (figure 2, lane 4). However, the extent of the eIF-2 phosphorylation in both cases was significantly less when compared to the extent of phosphorylation of added eIF-2 by exogenously added heme-regulated protein synthesis inhibitor (HRI) (figure 2, lane 1). We should point out, however, that in the absence of exogenously added eIF-2, we did not detect any significant phosphorylation of eIF-2 either in heme-deficient reticulocyte lysate or in heme-supplemented lysate with viral core although under both conditions the protein synthesis activity of the lysate was strongly inhibited. Apparently, the low sensitivity of our assay procedure precluded detection of eIF-2 phosphorylation under these conditions.

An indirect evidence for viral core-induced eIF-2  $\alpha$ -subunit phosphorylation was also obtained by assaying the GEF activity in an aliquot of reticulocyte lysate used for protein synthesis. It has been reported that eIF-2  $\alpha$ (P) formed by phosphorylation of eIF-2  $\alpha$ -subunit by HRI binds to GEF and inactivates it. As shown in figure 3, the reticulocyte lysate used for protein synthesis activity promotes [<sup>3</sup>H]-GDP displacement from exogenously added eIF-2·[<sup>3</sup>H]-GDP in the presence of unlabelled GDP and this GDP displacement activity was almost completely lost upon prior incubation of the lysate with vaccinia viral core presumably by inactivation of endogenous GEF by eIF-2  $\alpha$ (P) formed during incubation with the viral core.

## Discussion

Vaccinia viral core inhibits protein synthesis and also Met-tRNA<sub>f</sub>40S complex formation with physiological mRNAs in reticulocyte lysate (Ghosh-Dastidar *et al.*, 1981). In this paper, we provide evidence that vaccinia viral core promotes limited eIF-2  $\alpha$ -subunit phosphorylation when incubated in the presence of reticulocyte lysate and exogenously added eIF-2 and also inhibits the endogenous GEF activity in reticulocyte lysate. The viral core induced eIF-2  $\alpha$ -subunit phosphorylation may be the cause of viral core induced protein synthesis inhibition in reticulocyte lysate. Several observations support this hypothesis. First, eIF-2

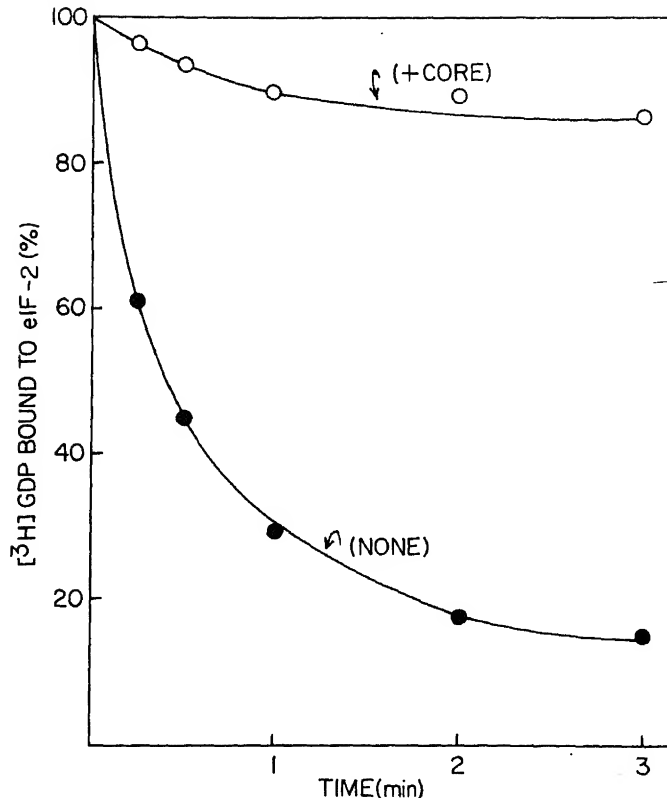




**Figure 2.** Phosphorylation of eIF-2  $\alpha$ -subunit.

The reaction mixtures contained 15  $\mu$ l reticulocyte lysate, 75  $\mu$ M KCl, 37  $\mu$ M Hemin-Cl, 8  $\mu$ g eIF-2 and 0.11  $A_{260}$  unit of vaccinia viral core as indicated. The reaction mixtures were incubated at 24°C for 10 min and were then mixed with (final vol, 45  $\mu$ l), 37.5  $\mu$ M amino acid mixture, 0.15 mM GTP, 7.5 mM creatine phosphate 2.25 U creatine phosphokinase, 10 mM Tris-HCl, pH 7.8. The reaction mixtures were then incubated at 30°C for 40 min. At all stages the reaction mixture contained 0.5  $\mu$ M [ $\gamma$ - $^{32}$ P]-ATP (sp. activity  $10^5$  cpm/pmol) and 1.5 mM Mg(OAc) $_2$ . For *in vitro* eIF-2 phosphorylation by HRI (Lane 1) 8  $\mu$ g eIF-2 was incubated with 3  $\mu$ g HRI, [ $\gamma$ - $^{32}$ P]-ATP (25  $\mu$ M; specific activity 2000 cpm/pmol) and 1.5 mM Mg(OAc) $_2$  at 37°C for 10 min. The reaction mixtures were then analyzed by NaDodSO $_4$ /polyacrylamide gel electrophoresis and autoradiography. Lane 1, eIF-2 + HRI + ATP; Lane 2, eIF-2 + Heme-supplemented lysate; Lane 3, -eIF-2 - Heme-supplemented lysate; Lane 4, +eIF-2 + Heme-supplemented lysate - core; Lane 5, +eIF-2 + Hemin supplemented lysate + core.

(Siekierka *et al.*, 1985; Van Steeg *et al.*, 1984), heat shock conditions (Duncan and Hershey, 1984; DeBenedetti and Baglioni, 1986) and also under different stress conditions (Duncan and Hershey, 1985). In each case, such phosphorylation is accompanied by reduced protein synthesis, or preferential synthesis of specific proteins.



**Figure 3.** Inhibition of GEF activity in reticulocyte lysate by vaccinia viral core.

The reaction mixture was preincubated at 25°C for 10 min with 150  $\mu$ l of reticulocyte lysate, 75 mM KCl, 37  $\mu$ M Hemin-Cl. Vaccinia viral core (50  $\mu$ l, 0.55 A<sub>260</sub> unit) were added as indicated. The reaction mixtures were then mixed with 37.5  $\mu$ M amino acid mixture 21 U creatine phosphokinase, 0.75 mM ATP, 0.15 mM GTP, 7.5 mM creatine phosphate, 1.5 mM (Mg OAc)<sub>2</sub>, 10.0 mM Tris-HCl, pH 7.8 and 75 mM KCl and incubated for 40 min at 30°C. The lysate mixture (425  $\mu$ l) was then diluted 1:1 with lysate dilution buffer containing 40 mM Tris-HCl (pH 7.8), 100 mM KCl, 50 mM KF, 10% Glycerol and 100  $\mu$ M unlabelled GDP. eIF-2-[<sup>3</sup>H]-GDP was preformed (5 min, 37°C) in a separate reaction mixture containing 25 mM Tris-HCl (pH 7.8), 90 mM KCl, 2.5 mM dithiothreitol, 160  $\mu$ g/ml bovine serum albumin, 1.5  $\mu$ M [<sup>3</sup>H]-GDP (5400 cpm/pmol) and eIF-2 (30  $\mu$ g/ml). Mg(OAc)<sub>2</sub> was then added to a final concentration of 1 mM. 150  $\mu$ l of preformed eIF-2-[<sup>3</sup>H]-GDP was incubated with 600  $\mu$ l of diluted lysate at 37°C. At indicated time 100  $\mu$ l sample was withdrawn and immediately added to 3 ml of ice-cold wash buffer containing 20 mM Tris-HCl (pH 7.8), 100 mM KCl, and 1 mM Mg(OAc)<sub>2</sub> to stop the reaction and the [<sup>3</sup>H]-GDP release was determined by standard Millipore filtration method.

adenovirus infected cell-free extracts, Siekierka *et al.* (1985) have reported that the GEF activity is responsible for reversal of protein synthesis inhibition caused by eIF-2  $\alpha$ -subunit phosphorylation by eIF-2 kinase (Reichel *et al.*, 1985). In this work, we have provided evidence that GEF activity is not responsible for reversal of vaccinia viral core induced protein synthesis inhibition in reticulocyte lysate. We

deficient reticulocyte lysate. An active RF preparation contains an excess eIF-2  $\alpha$ -subunit. We have postulated that RF provides this excess eIF-2  $\alpha$ -subunit to eIF-2  $\alpha$ (P) and thus reconstitute active eIF-2 molecules (Grace *et al.*, 1984). We believe, a similar mechanism is operative in RF promoted reversal of vaccinia viral core induced protein synthesis inhibition in reticulocyte lysate. The mechanism of eIF-2 action in reversal of viral core induced protein synthesis inhibition is not apparent. This reversal activity was observed with a partially purified eIF-2 preparation (Fraction III) and this activity was lost upon further purification. Several possibilities may be considered: (i) The reversal activity may be due to some contaminating factor which is lost upon further fractionation. (ii) The reversal activity is due to eIF-2 in association with other component(s) and this component(s) is lost upon further fractionation. (iii) eIF-2 is active in a specific conformation and this conformation is altered upon further fractionation.

## Acknowledgements

This research work was supported by a research grant from the Cancer and Smoking Disease Research Program LB 506, Nebraska Department of Health, University of Nebraska Biomedical Research Grant and NIH Research Grant GM 22079.

## References

- Amsez, H., Golmand, S. H., Haudrich-Morre, T., Voorma, H. O. and Benne, R. (1979) *Eur. J. Biochem.*, **98**, 513.
- Bablanian, R. (1975) *Prog. Med. Virol.*, **19**, 40.
- Bablanian, R., Esteban, B., Baxt, B. and Sonnadeno, J. A. (1978) *J. Gen. Virol.*, **39**, 391.
- Bagchi, M. K., Chakravarty, I., Ahmad, M. F., Nasrin, N., Banerjee, A. C., Olson, C. and Gupta, N. K. (1984) *J. Biol. Chem.*, **260**, 6950.
- Ben Hamida, F. and Beaud, G. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 175.
- Centralla, M. and Lucas-Lennard, J. (1982) *J. Virol.*, **41**, 781.
- Chakrabarti, D., Nasrin, N., Ramaiah, K. V. A., Bagchi, M. K. and Gupta, N. K. (1985). *13th International Congress of Biochemistry*, Amsterdam, The Netherlands.
- Chakravarty, I., Bagchi, M. K., Roy, R., Banerjee, A. C. and Gupta, N. K. (1985) *J. Biol. Chem.*, **260**, 6945.
- Coppola, G. and Bablanian, R. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 75.
- Dalls, S. (1965) *J. Cell. Biol.*, **18**, 51.
- Das, A., Bagchi, M. K., Ghosh-Dastidar, P. and Gupta, N. K. (1982) *J. Biol. Chem.*, **257**, 1282.
- DeBenedetti, A. and Baglioni, C. (1986) *J. Biol. Chem.*, **261**, 338.
- Dratewka-Kos, E., Kiss, I., Lucas-Lennard, J., Mehta, H. B., Woodley, C.L. and Wahba, A. J. (1984) *Biochemistry*, **23**, 6184.
- Duncan, R. and Hershey, J. W. B. (1984) *J. Biol. Chem.*, **259**, 11882.
- Duncan, R. and Hershey, J. W. B. (1985) *J. Biol. Chem.*, **260**, 5493.
- Edery, I., Humbelin, M., Darveau, A., Lee, K. A. W., Milburn, S., Hershey, J. W. B., Trachsel, H. and Sonnenberg, N. (1983) *J. Biol. Chem.*, **258**, 11398.
- Ghosh-Dastidar, P., Yaghamai, B., Das, A., Das, H. K. and Gupta, N. K. (1980) *J. Biol. Chem.*, **255**, 365.
- Ghosh-Dastidar, P., Goswami, B. B., Das, A., Das, P. and Gupta, N. K. (1981) *Biochem. Biophys. Res. Commun.*, **99**, 946.
- Grace, M., Ralston, R. O., Banerjee, A. C. and Gupta, N. K. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6517.
- Grace, M., Bagchi, M. K., Ahmad, M. F., Yeager, T., Olson, C., Chakravarty, I., Nasrin, N., Banerjee, A. C. and Gupta, N. K. (1984) *Proc. Natl. Acad. Sci. USA*, **79**, 6517.

- lik, W. K. (1962) *Virology*, **18**, 9.
- lik, W. K. (1969) *J. Mol. Biol.*, **8**, 262.
- mpfer, R. (1982) in *Protein Biosynthesis in Eukaryotes* (ed. R. Perez-Bercoff) (New York and London: Plenum Press) p. 223.
- niecny, A. and Safer, B. (1983) *J. Biol. Chem.*, **258**, 3402.
- mmli, U. K. (1970) *Nature (London)*, **227**, 680.
- umdar, A., Dasgupta, A., Chatterjee, B., Das, H. K. and Gupta, N. K. (1979) *Methods Enzymol.*, **60**, 35.
- ts, R. L., Levin, D. H. and London, J. M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2559.
- ss, B. (1968) *J. Virol.*, **2**, 1028.
- ss, B. and Filler, R. (1970) *J. Virol.*, **5**, 99.
- ins, J. R. and Joklik, W. K. (1977) *J. Biol. Chem.*, **252**, 6930.
- on, A., Ben Hamida, F. and Beaud, G. (1980) *Nature (London)*, **287**, 355.
- ton, R., Das, A., Grace, M., Das, H. K. and Gupta, N. K. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5490.
- u, R. S. and London, I. M. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **36**, 868 (Abstr).
- hel, P., Merrick, W. C., Siekierka, J. and Mathews, M. B. (1985) *Nature (London)*, **313**, 196.
- emond-Horntseak, H. and Moss, B. (1975) *J. Virol.*, **16**, 34.
- rom, M. and Bablanian, R. (1979) *Virology*, **99**, 319.
- ekin, A. J. (1965) *Nature (London)*, **199**, 357.
- ierka, J., Mitsui, K. and Ochoa, S. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 220.
- ierka, J., Mauser, L. and Ochoa, S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2537.
- ierka, J., Manne, V., Mauser, L. and Ochoa, S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1232.
- ierka, J., Mariano, T. M., Reichel, P. A. and Mathews, M. B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 959.
- Steege, H., Kasperatits, M., Voorma, H. O. and Benne, R. (1984) *Eur. J. Biochem.*, **138**, 473.



# Effect of proteolytic digestion on the function of vesicular stomatitis virus ribonucleoprotein complex

A. K. BANERJEE, J. ROY and D. J. CHATTOPADHYAY

Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 071 10, USA

**Abstract.** The nucleocapsid protein (49 Kd) of vesicular stomatitis virus is tightly bound to the genome rendering the latter transcriptionally competent. Controlled digestion with chymotrypsin removed a 12 Kd peptide from the complex. The resulting complex failed to serve as template for genome transcription *in vitro* when the polymerase components L and NS proteins were added. A template-associated protein kinase activity was also lost upon chymotrypsin treatment. However, the cleaved nucleocapsid protein (37 Kd) was still capable of binding tightly with the genome template and retained the epitope recognized by a monoclonal antibody. These results suggest that the nucleocapsid protein possesses separate domains that mediate binding to polymerase complex and maintain the structural integrity of the template.

**Keywords.** Vesicular stomatitis virus; transcription; regulation.

## Introduction

The transcribing ribonucleoprotein (RNP) complex of vesicular stomatitis virus (VSV) contains the negative strand genome RNA tightly associated with the nucleocapsid protein (N protein) (molecular weight, 49 Kd) and two other minor proteins, phosphoprotein NS (molecular weight, 29 Kd) and L protein (molecular weight, 241 Kd) (Wagner, 1975). The NS and the L proteins are easily dissociable from the N protein-RNA complex, rendering the latter transcriptionally inactive. Efficient reconstitution of *in vitro* transcription is achieved only when both the NS and the L proteins are added to the N protein-RNA template (Emerson and Yu, 1975; De and Banerjee, 1984). The transcription reconstitution, thus, has provided an excellent system to study the functions of individual polypeptide component in the *in vitro* transcription process. Using this system, we have recently shown that the L protein initiates RNA synthesis on the N protein-RNA template and the NS protein mediates chain elongation of RNA chains (De and Banerjee, 1985). Moreover, the requirements of L and NS proteins for optimal RNA synthesis *in vitro* are catalytic and stoichiometric, respectively. The precise function of the nucleocapsid protein N, which maintains the structural integrity of the genome RNA, with respect to the transcription process is unknown. Its direct involvement in RNA synthesis was shown by the ability of a monoclonal antibody, against the N protein, to inhibit *in vitro* transcription process (De *et al.*, 1982). Moreover, studies on the specific requirements of the N-RNA complex for RNA synthesis by the L and NS proteins of different serotypes showed a direct role of N protein in RNA synthesis (De and Banerjee, 1984).

In this communication we have subjected purified N-RNA complex to controlled

role in the *in vitro* transcription process.

## Materials and methods

### *Purification of VSV*

VSV (Indiana, Mudd-Summers Strain) grown in roller bottles containing monolayers of baby hamster kidney cells (BHK-21) and purified as previously described (Banerjee *et al.*, 1974). Using [ $^{35}\text{S}$ ]-methionine and [ $^3\text{H}$ ]-uridine during infection, VSV was labelled in protein and the genome RNA moieties, respectively.

### *Isolation and purification of L + NS proteins*

Purified VSV (4 mg at 500  $\mu\text{g}/\text{ml}$ ) was disrupted in 10 mM Tris-HCl, pH 8.0, containing 5% (v/v) glycerol, 0.4 M NaCl, 1.85% Triton-X100, and 0.6 mM dithiothreitol (final concentrations) (buffer A). The RNP was sedimented by centrifugation in an SW60 rotor at 200,000 *g* for 2 h, through 30% (v/v) glycerol onto a 100% glycerol cushion. The RNP was collected from the cushion and diluted 5 fold with buffer containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. An equal volume of 2X buffer A containing 0.8 M NaCl (without Triton) was added and kept in ice for 1 h. The mixture was centrifuged through 30% glycerol as described above. The released L and NS proteins were recovered from the top (approximately 2 ml) of the centrifuge tube, dialyzed against buffer 10 mM Tris-HCl, pH 8, 10% glycerol, 0.2 M NaCl and 0.3 mM dithiothreitol for 12 h at 4°C. The dialysate was concentrated by Amicon concentrator. The final protein concentration of L + NS was 630  $\mu\text{g}/\text{ml}$ . The purified L + NS proteins was kept in  $-70^\circ\text{C}$  before use.

### *Purification of N-RNA template*

N-RNA template was collected in a glycerol cushion after removal of the L and NS proteins by 0.8 M NaCl, as described above. The template was treated again with buffer A containing 0.8 M NaCl and recovered by centrifugation through 15% (w/v) Renografin solution and collected on a 76% Renografin cushion. The centrifugation was carried out in an SW60 rotor at 200,000 *g* for 2 h. The template was collected from the cushion and diluted to 4 ml by addition of buffer containing 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA with 1.4 g of CsCl and further centrifuged for 24 h in an SW60 rotor at 200,000 *g*. The template band was collected and dialyzed against 10 mM Tris-HCl, pH 8 and 50 mM NaCl, at room temperature. The dialysate was further concentrated by pelleting through 20% glycerol on a 100% glycerol cushion. The template was recovered and kept at  $-70^\circ\text{C}$ . The final concentration of N-RNA template was 560  $\mu\text{g}/\text{ml}$ .

### *Treatment of the template with chymotrypsin A*

The N-RNA template was treated with chymotrypsin A in a ratio of 4:25:1 (w/w)

chymotrypsin was added in an 8000 rotor at 15,000 rpm for 1 h, collected from the glycerol cushion (100%), and kept at  $-70^{\circ}\text{C}$ . The final concentration of treated N-RNA template was 350  $\mu\text{g}/\text{ml}$ .

### *In Vitro transcription-reconstitution reaction*

Purified N-RNA template or chymotrypsin-treated template were used to reconstitute RNA synthesis *in vitro* using purified L and NS proteins as detailed by De and Banerjee (1985). The  $\alpha$ - $^{32}\text{P}$  CMP labelled-RNA products were analyzed either by electrophoresis on a 20% polyacrylamide gel to detect the leader RNA or 5% polyacrylamide gel to separate individual VSV mRNA species (De and Banerjee, 1985).

### *Western blot*

The chymotrypsin-treated or untreated N-RNA template was electrophoresed on a 10% sodium dodecyl sulphate-polyacrylamide gel (Laemmli, 1970) and the proteins were transferred to nitrocellulose paper (S and S) and treated with the monoclonal antibody to N protein (De *et al.*, 1981) according to the method of Burnette (1982). The blot was then treated with the secondary antibody, goat anti-mouse immunoglobulin-G (IgG) followed by antibody to swine-antigoat IgG conjugated to horseradish peroxidase. The proteins were visualized by the color reaction with the substrate 4-chloro-1-naphthol.

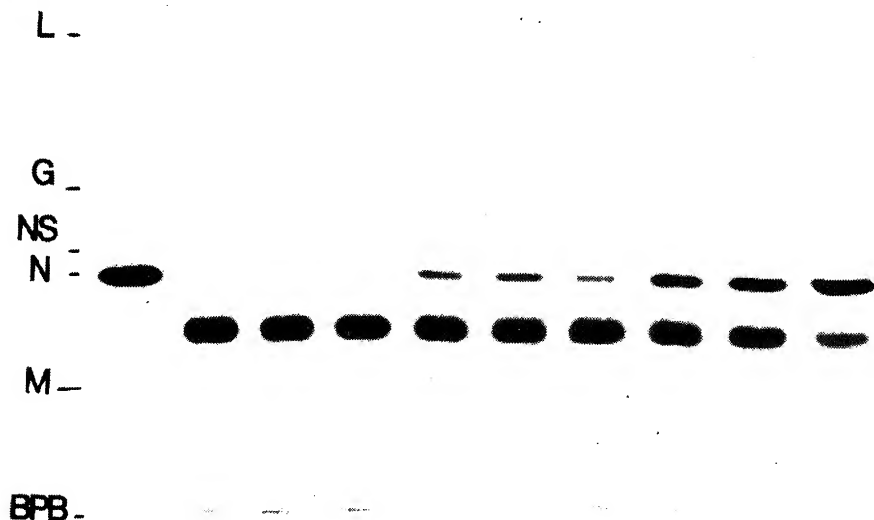
### *Protein kinase assay*

The reaction mixture contained chymotrypsin-treated or untreated N-RNA template (27  $\mu\text{g}$ ), Tris-HCl, pH 8.0, purified NS protein (1  $\mu\text{g}$ ), and 10  $\mu\text{C}$   $\gamma$ - $^{32}\text{P}$  ATP (3000 C/mmol). The reaction was at  $37^{\circ}\text{C}$  for 90 min. The phosphorylated protein products were analyzed by electrophoresis on 9% urea-SDS-polyacrylamide gel as detailed by Masters and Banerjee (1986).

## **Results and discussion**

$^{35}\text{S}$ -labelled purified VSV was used to prepare N-RNA template in which the N protein was exclusively labelled at methionine residues. By varying the concentrations of chymotrypsin and the N-RNA template and incubation for different periods of time, the proteolytic digestion of the N protein within the complex was monitored by electrophoresis on 10% polyacrylamide gel. As shown in figure 1, a specific cleavage of the N protein occurred when the ratio of the template to chymotrypsin was 4.25:1 (w/w) and the incubation time was 20 min at  $37^{\circ}\text{C}$  (lane 2). The molecular weight of the cleaved N protein was approximately 37 Kd, indicating that a 12 Kd peptide must have been removed during the proteolytic digestion. In all other combinations and times of incubation, the digestions were incomplete. It should be noted that recovery of the radioactivity in the N protein following digestion was about 90%.



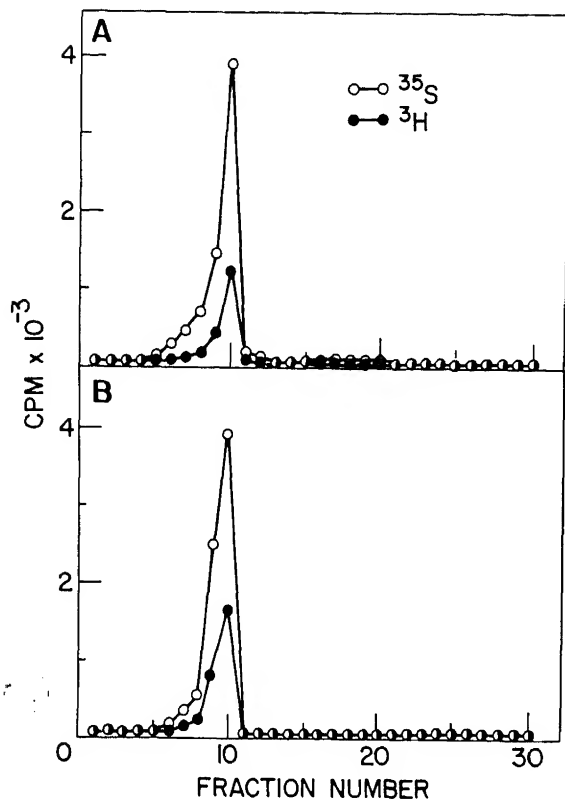


**Figure 1.** Effect of chymotrypsin on VSV N protein-RNA template. [ $^{35}\text{S}$ ]-labelled template RNA was purified as described in 'materials and methods' and treated with chymotrypsin at a ratio (w/w) of template to chymotrypsin 4:25:1 and incubated at 20 min (lane 2), 40 min (lane 3), 60 min (lane 4), and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Template to chymotrypsin ratio of 8:5:1 was used for 20 min (lane 5), 40 min (lane 6) and 60 min (lane 7) and 17:1 was used for 20 min (lane 8), 40 min (lane 9) and 60 min (lane 10). Lane 1 served as control without chymotrypsin treatment. The migration positions of VSV structural protein L, G, NS, N and M are shown. Autoradiography was carried out using Kodak XAR2 film. BPB, Migration of bromophenol blue dye.

These results indicate that a small peptide (12 Kd) containing one or more methionine residues was cleaved from the N protein.

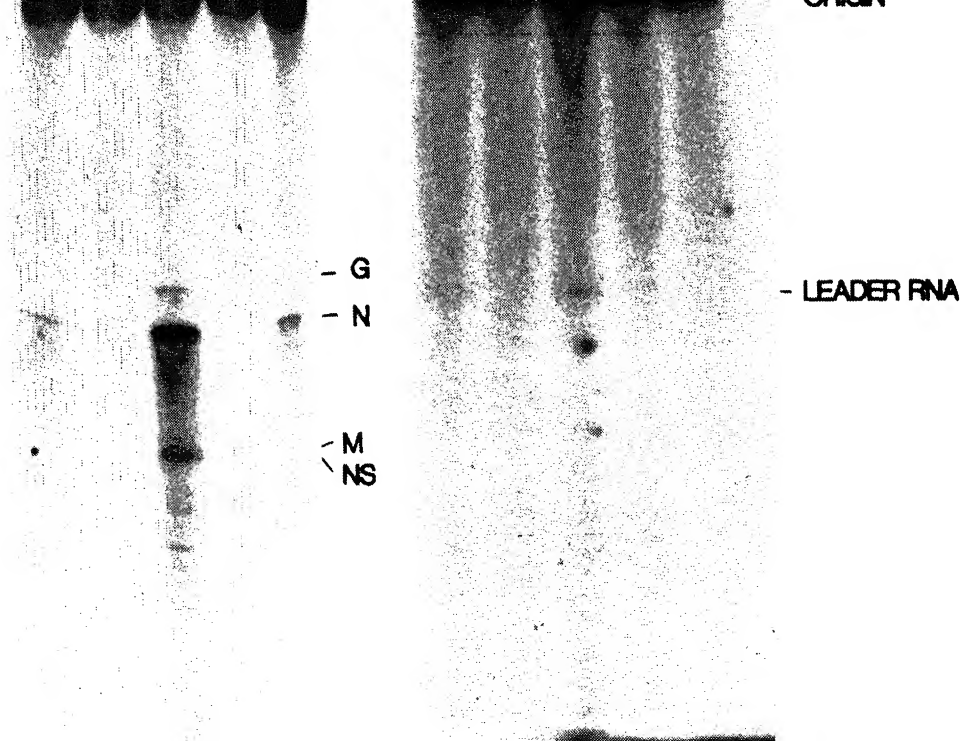
In order to study whether the cleavage of the N protein may have some adverse effect on its binding to the genome RNA, the chymotrypsin-treated  $^{35}\text{S}$  (protein) and  $^3\text{H}$ -U (genome)-labelled N-protein complex was banded in a CsCl gradient. As shown in figure 2, both the radioactivities in treated (B) and the untreated control (A) sedimented at the same position. These results indicate that the cleaved N protein was associated with the genome RNA strongly enough such that the complex was not disrupted by CsCl during the prolonged time of centrifugation. Thus, it seems that the cleaved part of the N protein may not be necessary for the tight association of the N protein with the genome template.

Although the structural integrity of the N-RNA template remained intact after chymotrypsin digestion, it was important to study whether the cleavage of the



**Figure 2.** CsCl sedimentation of <sup>35</sup>S and <sup>3</sup>HU-labelled N-RNA template following digestion with chymotrypsin. The untreated control (panel A) and treated (panel B) templates were centrifuged in a CsCl gradient as detailed in 'materials and methods'. Aliquots were collected from the top and radioactivity in each fraction was determined.

complex was retained in a transcription-reconstitution reaction. *In vitro* transcription was carried out using chymotrypsin-treated template with saturating concentrations of purified L and NS proteins. The products were analyzed by electrophoresis on 20% and 5% polyacrylamide gels. As shown in figure 3, synthesis of full-length NAs (lane 5) and leader RNA (lane 10) were reduced by more than 95% compared to the control (lanes 3 and 8). These results clearly indicate that the ability of the N-RNA complex to act as template for transcription was virtually lost after treatment with chymotrypsin. It is important to mention that to carry out the experiments described above, it was essential to confirm that the chymotrypsin-treated N-RNA complex was completely free of the proteolytic enzyme following purification of the template by CsCl banding or pelleting in glycerol cushion. Any contaminant chymotrypsin would digest the L and NS proteins during subsequent transcription-reconstitution reactions. We have routinely tested the recovered N-RNA template

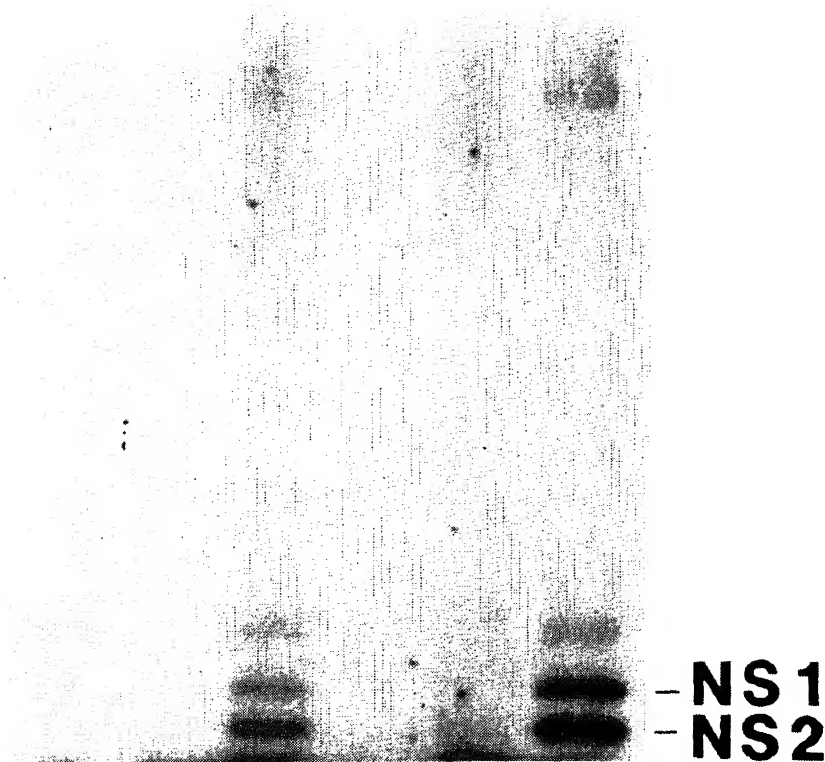


**Figure 3.** Analyses of the RNA products in a transcription-reconstitution reaction using treated and untreated templates.  $\alpha$ - $^{32}$ CMP-labelled RNA products synthesized using template reconstituted with purified L and NS proteins were analyzed by electrophoresis on 5% (lanes 1-5) and 20% (lanes 6-10) polyacrylamide gels. Lanes 1 and 6 contained L and NS alone; lanes 2 and 7 contained untreated N-RNA template alone; lanes 3 and 8 contained untreated N-RNA template+L+NS proteins; lanes 4 and 9 contained treated N-RNA template alone; lanes 5 and 10 contained treated N-RNA template+L+NS proteins. Migration positions of the VSV mRNAs coding for G, N, M and NS and leader RNA are shown.

*vitro* (Masters and Banerjee, 1986). To test whether chymotrypsin digestion also abrogates the protein kinase activity, the treated template was used in an *in vitro* kinase reaction using purified NS as the acceptor protein. As shown in figure 4, the template associated protein kinase activity was completely lost upon treatment with chymotrypsin, indicating that the enzyme activity is selectively affected, as is the template function, by proteolytic digestion. Note that two NS sub-species (NS1 and NS2) (Masters and Banerjee, 1986) were phosphorylated by the template-associated protein kinase.

Finally, we studied whether the cleaved N protein retained the antigenic site reactive to the monoclonal antibody raised against purified N protein. This monoclonal antibody is highly specific for N protein of Indiana serotype and does not

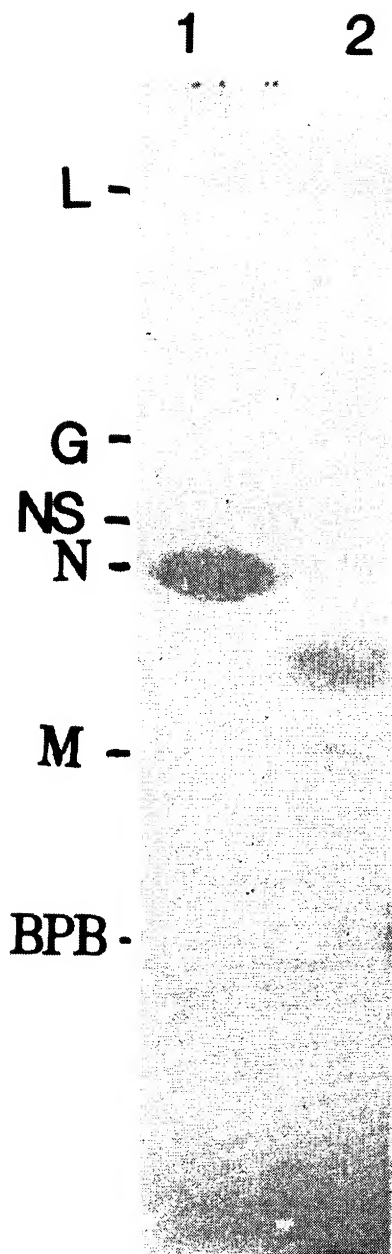
1 2 3 4 5 6



**Figure 4.** Protein kinase activity associated with N protein-RNA template. Chymotrypsin-treated and untreated templates were used in a protein kinase reaction with purified NS as the acceptor protein as detailed in 'materials and methods'. Lane 1, template only; lane 2, NS only; lane 3, 4, 5, NS protein + template treated with chymotrypsin for 0 min, 20 min, and 60 min, respectively; lane 6, untreated template + NS protein.

react with the corresponding protein of the New Jersey serotype (De *et al.*, 1982) in spite of the fact that both the N proteins share more than 80% homology in amino acid sequence (Banerjee *et al.*, 1984). As shown in figure 5, the proteolytically cleaved N protein reacted efficiently with the monoclonal antibody indicating that the epitope in the N protein recognized by the antibody is retained in the cleaved peptide fragment.

The above results clearly indicate that the N protein which is tightly associated with the genome template of VSV is directly involved in the genome transcription *in vitro*. Specific removal of a 12 Kd polypeptide from the N protein-RNA template by chymotrypsin has rendered the template transcriptionally incompetent as well as abrogated the associated protein kinase activity. Thus it seems that the cleaved portion of the N protein may contain the site for binding of the L and NS protein for initiation and elongation of RNA chains on the genome template. In addition, this portion may also contain the active site of the protein kinase activity. Alternatively, removal of the polypeptide segment may affect adversely on the overall secondary structure of the complex such that binding of the L and NS proteins and the protein



**Figure 5.** Western blot analysis of N protein with monoclonal antibody. The detail of the electrophoresis of the N protein, blotting, and antibody complex formation and subsequent color reaction are detailed in 'materials and methods'. Lane 1, untreated N; lane 2, chymotrypsin-treated N. Migration positions of VSV structural proteins are shown.

kinase activity are all compromised. However, the N protein after chymotrypsin treatment remained tightly bound to the genome template and the epitope recognized by a monoclonal antibody is also retained. Thus, it seems that there are at least two domains in the N protein; one that contains the binding site for the polymerase and the other mediates the interaction with genome RNA to impart the helical structure. The former domain is susceptible to proteolytic cleavage, whereas the latter one is tightly associated with the helical structure and is resistant to such digestion and contains the site reactive to monoclonal antibody. Preliminary results indicate that the N-terminal end of the 37 Kd N protein is unblocked, indicating that the N-terminal portion (which contains blocked N-terminus) of the N protein has been removed. Determination of the N-terminal amino acid sequence of 37 Kd N protein, in progress, should precisely locate the site of proteolytic cleavage within the N protein. Further studies are in progress to relate these domains to specific functions of the N protein.

## References

- Banerjee, A. K., Moyer, S. A. and Rhodes, D. P. (1974) *Virology*, **61**, 547.  
Banerjee, A. K., Rhodes, D. P. and Gill, D. P. (1984) *Virology*, **137**, 432.  
Burnette, W. N. (1982) *Anal. Biochem.*, **124**, 396.  
De, B. P. and Banerjee, A. K. (1984) *J. Virol.* **51**, 628.  
De, B. P. and Banerjee, A. K. (1985) *Biochem. Biophys. Res. Commun.*, **126**, 40.  
De, B. P., Tahara, S. and Banerjee, A. K. (1982) *Virology*, **122**, 510.  
Emerson, S. U. and Yu, Y-H. (1975) *J. Virol.*, **15**, 1348.  
Laemmli, U. K. (1970) *Nature (London)*, **227**, 680.  
Masters, P. S. and Banerjee, A. K. (1986) *Virology*, **154**, 259.  
Sánchez, A., De, B. P. and Banerjee, A. K. (1985) *J. Gen. Virol.*, **66**, 1025.  
Wagner, R. R. (1975) *Compr. Virl.*, **4**, 1.



## Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets

ASOK BANERJEE, SANKAR N. MAITY, SUKLA RAY CHAUDHURI  
and B. BHATTACHARYYA\*

Department of Biochemistry, Bose Institute, Calcutta 700 054, India.

**Abstract.** Colchicine-tubulin dimer complex, a potent inhibitor of normal microtubule assembly undergoes extensive self-assembly in the presence of  $1 \times 10^{-4}$  M zinc sulphate. Polymers assembled from colchicine-tubulin dimer complexes are sensitive to cold.

Although colchicine can be accommodated within the polymeric structure, the drug cannot bind to tubulin subunits in the intact polymers. This is evidenced by the fact that (a) the colchicine binding activity of tubulin is lost when allowed to polymerize with zinc sulphate, (b) the loss in colchicine binding could be prevented by preincubation of tubulin with  $1 \times 10^{-3}$  M  $\text{CaCl}_2$  or  $1 \times 10^{-5}$  M vinblastine sulphate and finally (c) no loss in colchicine binding activity is found when tubulin is kept at a concentration far below the critical concentration for polymerization. Unlike colchicine, its B-ring analogues desacetamido colchicine (devoid of the B-ring substituent) and 2-methoxy-5- (2',3',4'-trimethoxyphenyl) tropone (devoid of the B-ring) can bind to tubulin subunits in the intact polymers.

Thus we conclude that the colchicine binding domain on the tubulin molecule is mostly (if not completely) exposed in the Zn(II)-induced polymers and the B-ring substituent plays a major role in determining the binding ability of a colchicine analogue to tubulin in the intact Zn(II)-induced sheets.

**Keywords.** Colchicine-tubulin dimer complex; B-ring; tubulin sheets.

## Introduction

The alkaloid colchicine was known as an inhibitor of mitosis long before the discovery of microtubules (Dustin, 1978). Extensive studies on the mechanism of action of colchicine have established that it exerts its antimitotic action through binding to tubulin. Although tubulin can bind colchicine, however, tubulin within the assembled microtubule cannot bind colchicine (Wilson and Meza, 1973). *In vitro* microtubule assembly is inhibited by substoichiometric concentrations of colchicine; half maximal inhibition occurs when only 2% of the unpolymerized tubulin is complexed with drug (Olmsted and Borisy, 1973; Wilson and Bryan, 1974; Margolis and Wilson, 1977). A similar mechanism also appears to be applicable to *in vivo* poisoning; in the case of mitosis, colchicine exerts its influence when only a small fraction of the cellular pool of tubulin is complexed with drug.

This substoichiometric poisoning of microtubule assembly has been explained by the Capping of the growing end of microtubules by the drug-tubulin complex (Margolis and Wilson, 1977). Sternlicht and Ringel (1979) explained the above phenomena on the basis of a copolymerization model in which both tubulin and colchicine-tubulin dimer (CD) complex are incorporated into microtubules.



According to their model, incorporated CD complexes decrease the affinity of the microtubule end for further addition of tubulin dimer. It has been reported from this laboratory (Banerjee *et al.*, 1982) that even only CD complexes could be polymerized in the presence of zinc sulphate. Analysis of the polymerized products harvested in the presence of [ $^3\text{H}$ ]-colchicine indicates the incorporation of 0.5 mol of colchicine per mol of tubulin. From this observation, it is apparent that the colchicine binding site on tubulin might be different from the tubulin-tubulin interaction sites involved in the process of Zn(II)-induced assembly. Thus, the question may arise as to whether the colchicine binding site in the Zn(II)-induced polymer is buried, if not, whether colchicine could bind to intact Zn(II)-induced polymer. Our studies on this aspect revealed that the preformed sheets cannot bind colchicine. However, colchicine analogues with no substituents in the B-ring (of colchicine) (figure 7) such as desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (devoid of B-ring) could bind to intact Zn(II)-induced sheets suggesting that the size of the B-ring of colchicine plays an important role in determining the binding properties of colchicine to Zn(II)-induced sheets.

## Materials and methods

### Materials

Colchicine, Guanosine-5'-triphosphate (GTP) (Grade IIS), 2-(N-morpholino)ethanesulphonic acid (MES) and sodium dodecyl sulphate (SDS) were products of Sigma Chemical Company, St. Louis, Missouri, USA. Colcemid was obtained from K and K. B-ring modified analogues of colchicine were the kind gifts of Dr. Thomas J. Fitzgerald of School of Pharmacy, Florida A and M University, Tallahassee, Florida, USA. DEAE-cellulose (DE 52) and DEAE-cellulose paper (DE 81) were obtained from Whatman Ltd., England. [ $^3\text{H}$ ]-Colchicine (ring C, methoxy  $^3\text{H}$ ) was a product of New England Nuclear Corporation, USA. Fresh goat brains were obtained from a local slaughter house.

### Purification of tubulin

Tubulin was purified from goat brain in PMG buffer (10 mM potassium phosphate, pH 7.0; 10 mM  $\text{MgCl}_2$ ; 0.1 mM GTP) according to Weisenberg *et al.* (1968), except that DEAE-cellulose was used instead of DEAE-sephadex. The active fractions as judged from a colchicine-binding assay (Williams and Wolff, 1972) were pooled, concentrated by overnight dialysis at 0°C against 100 volumes of 8 M glycerol in buffer A (100 mM MES, pH 6.4; 0.5 mM  $\text{MgCl}_2$ ; 1.0 mM GTP) and stored at -70°C. The purity of tubulin was checked by SDS-polyacrylamide gel electrophoresis according to Laemmli (1970). Protein concentrations were determined as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

the cuvette chamber was maintained at 37°C using an LKB model 2209 circulator.

### *[<sup>3</sup>H]-Colchicine-binding assay*

[<sup>3</sup>H]-Colchicine binding assay was performed according to the DEAE-cellulose filter disc method of Weisenberg *et al.* (1968) as modified by Williams and Wolff (1972).

### *Fluorometric assay for drug binding*

The binding of unlabelled drug (colchicine, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone) to tubulin was assayed by the fluorometric method according to Bhattacharyya and Wolff (1974). Fluorescence was measured at 37°C in a Perkin Elmer model MPF 44B Spectrofluorometer. Samples were excited at a wavelength of 380 nm, emission was scanned from 390–550 nm.

### *Electron microscopy*

Electron microscopy was done according to Johnson and Borisy (1979) using 1% uranyl acetate.

## **Results**

### *Colchicine binding activity of tubulin upon assembly in the presence of Zn(II)*

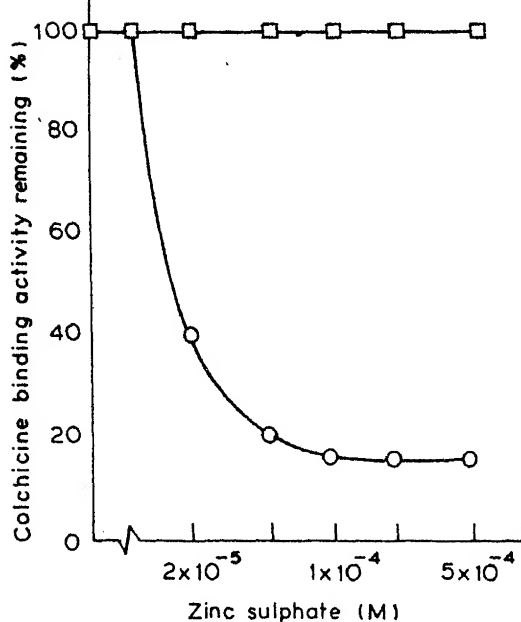
It has been demonstrated from this laboratory that suprapstoichiometric concentrations of colchicine do not inhibit the assembly of tubulin in the presence of zinc sulphate (Banerjee *et al.*, 1982). We also demonstrated that even CD complexes are capable of assembly in the presence of  $1 \times 10^{-4}$  M zinc sulphate. Electron microscopic examination of the assembly products obtained from pure tubulins by Zn(II) are shown in figure 1. Negatively stained samples showed that at the highest Zn(II) concentration ( $1 \times 10^{-4}$  M) the polymers are sheets in nature. This is in agreement with the results of others (Larsson *et al.*, 1976; Wallin *et al.*, 1977; Gaskin and Kress, 1977; Haskins *et al.*, 1980). The colchicine content of the Zn(II)-induced polymers harvested from the [<sup>3</sup>H]-labelled CD complexes have been determined and found to be 0.4–0.5 mol of colchicine per mol of tubulin dimer. It appears from this that the colchicine-binding site and the tubulin-tubulin interaction sites are different in the case of Zn(II)-induced assembly. The incorporation of the colchicine molecule into the Zn(II)-induced polymer further indicates that the polymer could accommodate the drug molecule within its lattice. Thus, the question may arise as to whether the preformed polymers bind colchicine. Therefore, experiments were designed to study the colchicine-binding property of 6S tubulin after assembly in the presence of zinc sulphate.

For this purpose, aliquots of tubulin were incubated with various concentrations of zinc sulphate at 37°C for 30 min; samples were subsequently incubated further with [<sup>3</sup>H]-colchicine ( $1 \times 10^{-6}$  M) at 37°C for 30 min and the bound colchicine was



**Figure 1.** Electron micrographs of Zn(II)-tubulin polymer. DEAE-tubulin (0.7 mg/ml) in buffer A was polymerized at 37°C for 30 min in the presence of  $1 \times 10^{-4}$  M  $\text{ZnSO}_4$ . A 5  $\mu\text{l}$  aliquot of the polymerized sample was loaded onto a 400 mesh copper grid and negatively stained with 1% uranyl acetate as described in the 'materials and methods'. Grids were examined in a Phillips Model EM301 electron microscope under an accelerating voltage of 60 KV ( $\times 19,000$ ).

assayed (figure 2). As shown in figure 2 preincubation of tubulin with  $\text{ZnSO}_4$  at concentrations upto  $1 \times 10^{-5}$  M had no effect on the colchicine-binding activity while at concentrations above  $2 \times 10^{-5}$  M it caused a gradual inhibition of colchicine binding activity. The maximum inhibition occurred in the range of  $5 \times 10^{-5}$ – $1 \times 10^{-4}$  M  $\text{ZnSO}_4$  and further increase in the zinc sulphate concentration did not result in any further increase in the extent of inhibition. Infact, Zn(II) at  $1 \times 10^{-5}$  M does not induce assembly but the induction of assembly occurs at a Zn(II) concentration above  $2 \times 10^{-5}$  M (figure 3). It is, therefore, conceivable that the inhibition of colchicine binding activity of tubulin upon incubation with  $\text{ZnSO}_4$  is a consequence of polymerization of tubulin. That this inhibition in colchicine binding is not due to the blocking of sulphhydryl groups is evidenced by the fact that the inclusion of mercaptoethanol into the reaction mixture could not overcome the inhibition. That the inhibition is not due to the inactivation (or denaturation) of tubulin is shown by the total recovery of the colchicine-binding activity on treatment with Zn(II)-chelators like ethylenediamine tetraacetic acid or *o*-phenanthroline (data not shown). Moreover, this inhibition in colchicine binding could be prevented by incubation of tubulin with its assembly inhibitor such as  $1 \times 10^{-3}$  M calcium chloride or



**Figure 2.** Colchicine binding activity of tubulin upon assembly in the presence of  $\text{ZnSO}_4$ .

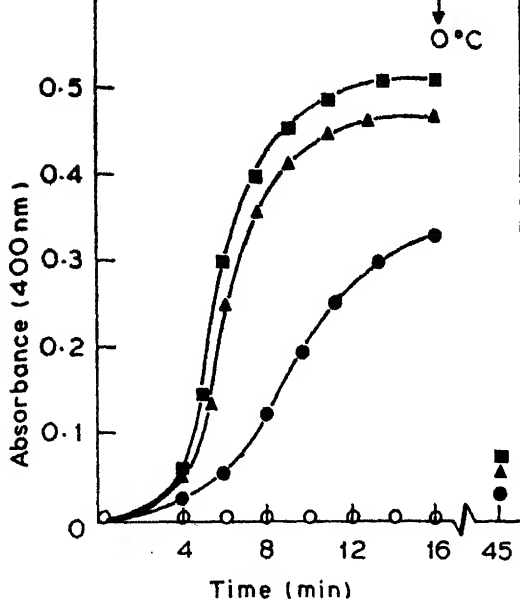
Aliquots of DEAE-purified tubulin (0.75 mg/ml) in buffer A were incubated with different concentrations of  $\text{ZnSO}_4$  at  $37^\circ\text{C}$  for 30 min. Colchicine binding activity of the samples were subsequently determined by incubating the samples with  $[^3\text{H}]$ -colchicine ( $1 \times 10^{-6}\text{M}$ ) at  $37^\circ\text{C}$  for 30 min. Colchicine binding assays were carried out with DE-81 filter paper as described under 'materials and methods'. Binding activity of each sample was expressed as a per cent activity remaining compared to a sample containing no  $\text{ZnSO}_4$  (O). A second set of samples was preincubated with either  $1 \times 10^{-3}\text{M}$   $\text{CaCl}_2$  (□) or  $1 \times 10^{-5}\text{M}$  vinblastine sulphate (□) prior to the incubation with  $\text{ZnSO}_4$ . For non polymerizing condition; aliquots of DEAE-tubulin (0.05 mg/ml) in buffer A were incubated with different concentration of  $\text{ZnSO}_4$  at  $37^\circ\text{C}$  for 30 min and the colchicine binding activity of the samples were determined (□) (taken from Banerjee, 1981).

binding of  $\text{Zn(II)}$  to tubulin. However, the latter possibility seems to be unlikely since  $\text{Zn(II)}$  exhibits no inhibitory effect when tubulin concentration was kept far below the critical concentration\* for polymerization (figure 2). Moreover, the loss in colchicine-binding activity has been found to correlate with the loss of tubulin from the supernatant (table 1). A slight variation in the results obtained by two different methods might result due to the binding of colchicine to ends of tubulin sheets. All these data clearly indicate that the intact  $\text{Zn(II)}$ -induced polymer binds colchicine very poorly.

#### *Studies with colchicine analogue*

From the previous results, it is clear that although the colchicine molecule could be accommodated with the  $\text{Zn(II)}$ -induced polymers, the drug binds to intact sheets very

\*Critical concentrations for polymerization of DEAE-purified tubulin in the presence and absence of



**Figure 3.** Assembly of DEAE-tubulin at different Zn(II) concentrations.

DEAE-tubulin (0.7 mg/ml) in buffer A was incubated at 37°C in the presence of different concentrations of ZnSO<sub>4</sub> and the polymerization was monitored turbidimetrically at 400 nm. ZnSO<sub>4</sub> concentrations were as follows: None or  $1 \times 10^{-5}$  M (○),  $2 \times 10^{-5}$  M (●),  $5 \times 10^{-5}$  M (▲) and  $1 \times 10^{-4}$  M (■). Arrow indicates the time when cuvette was placed at 0°C.

**Table 1.** Correlation of the colchicine binding inhibition with the amount of protein pelleted by centrifugation.

Tubulin concentration (mg/ml)	Colchicine binding <sup>a</sup>		Inhibition in colchicine binding (%)	Protein concentration (mg/ml)		
	Unpolymerized	Polymerized		Before polymerization	After pelleting down the polymer <sup>b</sup>	Protein pelleted (%)
1.2	5140	640	87.6	1.2	0.10	92
0.8	3749	580	84.6	0.8	0.11	87
0.4	2032	632	69	0.4	0.10	75

<sup>a</sup>Counts/min/0.1 ml reaction mixture. <sup>b</sup>Supernatants were examined for protein.

Aliquots of DEAE-purified brain tubulin were incubated at different protein concentrations in buffer A with or without  $1 \times 10^{-4}$  M ZnSO<sub>4</sub> at 37°C for 30 min. In one set of experiments, colchicine binding of samples were determined by further incubating the samples with [<sup>3</sup>H]-colchicine ( $1 \times 10^{-6}$  M) at 37°C for 30 min. In the other set of experiments, samples polymerized in the presence of ZnSO<sub>4</sub> were centrifuged at 120,000 *g* for 30 min at 25°C to pellet down the polymer.

poorly. Possible explanation for the burial of the colchicine-binding site in the Zn(II)-induced polymer might be that although the tubulin-tubulin interaction sites and the colchicine-binding sites in native tubulin are different, it is possible that the colchicine-binding site and the tubulin-tubulin interaction sites involved in sheet

formation may overlap spatially. An alternate mechanism might be that colchicine binding to tubulin which requires a change in conformation of the tubulin molecule (Ventilla *et al.*, 1972; Garland, 1978; Lambeir and Engelborghs, 1981; Detrich *et al.*, 1982; Andreu and Timasheff, 1982a) is hindered due to a restricted movement of tubulin in the assembled state compared to the free state.

To test these possibilities, we chose certain colchicine analogues *viz.*, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone which are known to bind tubulin at the same site where colchicine binds (Ray *et al.*, 1981). Further, they are structurally related to, yet less bulky than colchicine (figure 7).

*Effect of colchicine analogues on the Zn(II) tubulin sheet formation:* We have demonstrated that colchicine is ineffective at inhibiting the Zn(II)-tubulin sheet formation (Banerjee *et al.*, 1982). To test the effect of colchicine analogues on Zn(II)-tubulin sheet formation, we preincubated brain tubulin with  $1 \times 10^{-5}$  M of either analogue at 37°C for 30 min. Assembly was subsequently initiated by the addition of  $1 \times 10^{-4}$  M  $\text{ZnSO}_4$  (figure 4). As shown in the figure, tubulin complexed with desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone could polymerize well in the presence of  $\text{ZnSO}_4$ . In the case of the drug-treated samples the rate of assembly was less although the steady state turbidities were almost the same as that of the control sample. In this context it should be mentioned that similar behaviours of colchicine and podophyllotoxin in the assembly of microtubule protein in the presence of a GTP analogue, guanylyl-5'-methylene diphosphonate (pp ( $\text{CH}_2$ ))

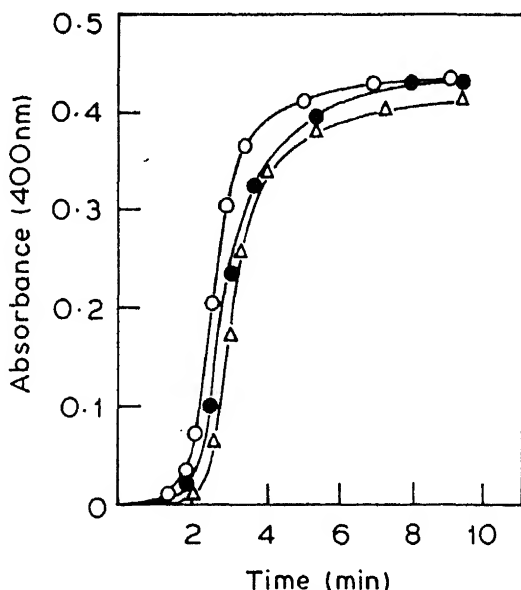


Figure 4. Effect of colchicine analogues on the Zn(II)-tubulin assembly.

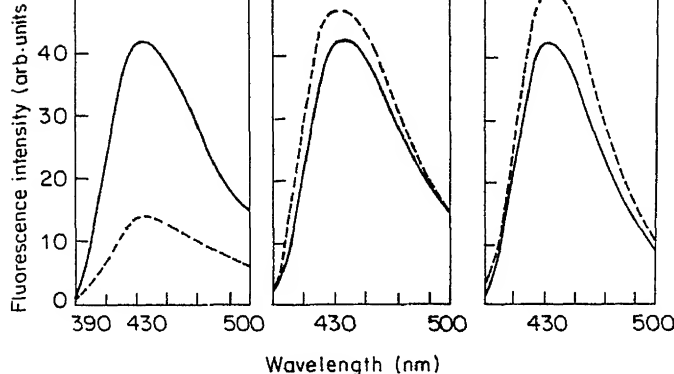
Aliquots of DEAE-purified brain tubulin (0.7 mg/ml) in buffer A were incubated at 37°C for 30 min either alone (○) or in the presence of  $1 \times 10^{-4}$  M desacetamido colchicine (△) or  $1 \times 10^{-4}$  M 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (●). Samples were subse-

polymers showed amorphous structure as well as large aggregates which appear to be fibrous and did not reveal any organized structure (Saltarelli and Pantaloni, 1982; Andreu and Timasheff, 1982a,b).

*Effect of colchicine analogues on the preformed Zn(II)-tubulin sheets:* In order to study the effect of colchicine analogues on the preformed Zn(II)-tubulin sheets, tubulin was assembled in the presence of  $1 \times 10^{-4}$  M  $\text{ZnSO}_4$  at  $37^\circ\text{C}$  for 30 min. Steady state polymers were further incubated with  $1 \times 10^{-4}$  M drug at  $37^\circ\text{C}$  and the turbidity was monitored. It has been observed that the turbidity remained almost unchanged for about an hour in the case of desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone treated samples whereas there are about 15–20% decrease in the optical density in the case of colchicine treated one. These results indicate that Zn(II)-tubulin sheets are stable to treatment with desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone whereas they are partially susceptible toward colchicine (data not shown).

*Binding of colchicine analogues to Zn(II)-tubulin sheets:* Unlike colchicine, its two B-ring analogues, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone bind to tubulin at a faster rate (Ray *et al.*, 1981). Like colchicine, the analogues upon binding to tubulin exhibit fluorescence emission maxima at 430 nm. To study the binding of these analogues to intact Zn(II)-induced polymers tubulin was assembled to steady state in the presence of zinc sulphate ( $1 \times 10^{-4}$  M) and subsequently incubated further with  $1 \times 10^{-4}$  M drug at  $37^\circ\text{C}$  for 15 min. The fluorescence spectra of the drug treated polymerized samples were scanned and compared with an identically treated sample of unpolymerized tubulin. The results of such a study employing colchicine, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone have been depicted in figure 5. As evident from the figure, there is a marked difference between the colchicine fluorescence of a sample of unpolymerized tubulin and that of an identical sample polymerized to steady state in the presence of zinc sulphate. The fluorescence of the polymerized sample is about 30–35% of that of the unpolymerized one (figure 5A). In fact, this 30–35% of fluorescence is due to the free tubulin after the colchicine-treatment of polymers. The total free tubulin is the sum of the tubulin present in equilibrium with the polymers at steady state (critical protein concentration for polymerization) plus the amount derived from the depolymerization of polymers by colchicine treatment ( $1 \times 10^{-4}$  M). This result is consistent with the conclusion obtained from figure 2 that the Zn(II)-induced polymers cannot bind colchicine. In contrast, the fluorescence emission spectra of both desacetamido colchicine (figure 5B) and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (figure 5C) were almost the same with either polymerized or unpolymerized sample of tubulin. These results clearly indicate that unlike colchicine, its two B-ring analogues could bind to intact Zn(II)-induced polymers of tubulin. In all cases, the fluorescence emission intensity was corrected for protein in the absence of drug.

The same conclusion is further confirmed by the  $[^3\text{H}]$ -colchicine binding assay. It



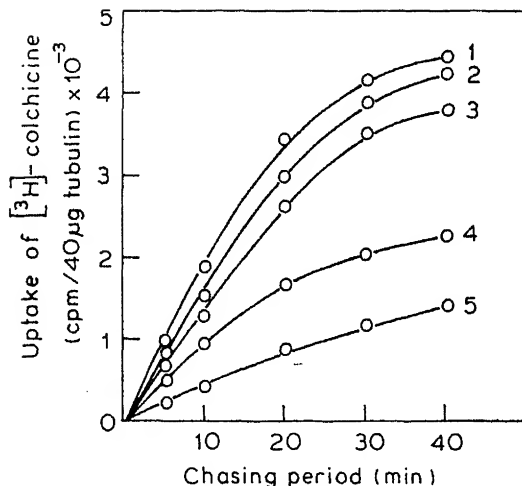
**Figure 5.** Binding of colchicine and its analogues to tubulin before and after assembly in the presence of  $\text{ZnSO}_4$ . Aliquots of DEAE-purified tubulin (0.98 mg/ml) in buffer A were polymerized in the presence of  $1 \times 10^{-4}$  M  $\text{ZnSO}_4$  at  $37^\circ\text{C}$  for 30 min. These polymerized samples together with nonpolymerized tubulin samples were incubated with  $1 \times 10^{-4}$  M drug at  $37^\circ\text{C}$  for 15 min. Fluorescence emission spectra of the samples were recorded subsequently from 390–500 nm after exciting at 350 nm. Fluorescence of both polymerized (---) and unpolymerized (—) samples are presented (A), Colchicine; (B), desacetamido colchicine; (C), 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone.

Appropriate corrections were made for scattering of assembled tubulin in the absence of drug (Banerjee, 1981).

has been reported from this laboratory (Ray *et al.*, 1981) that colcemid, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone bind to tubulin at the same site at which colchicine binds. Thus it has been possible to study the binding of these B-ring analogues to the intact Zn(II)-induced polymers in an experiment in which [ $^3\text{H}$ ]-colchicine could be used as a probe to detect the presence of drug molecules bound at the colchicine binding site on tubulin.

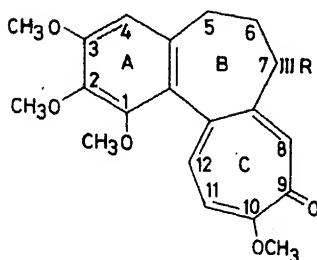
In this study, tubulin was polymerized in the presence of  $1 \times 10^{-4}$  M zinc sulphate to steady state and the polymers were subsequently incubated further with  $1 \times 10^{-4}$  M unlabelled drugs at  $37^\circ\text{C}$  for 30 min. The drug treated polymers were pelleted by warm ( $25^\circ\text{C}$ ) centrifugation at 120,000 *g* for 30 min. The pellets were resuspended in buffer A, depolymerized with EDTA and were incubated with [ $^3\text{H}$ ]-colchicine. The course of [ $^3\text{H}$ ]-colchicine binding was followed in each case and was compared to that of a control pellet obtained without treatment by any drug. The results of such an experiment employing colchicine, colcemid, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone have been summarized in figure 6. As shown in the figure, the uptake of [ $^3\text{H}$ ]-colchicine for colchicine and colcemid-treated polymer pellets are almost identical to that of the untreated control pellet, which clearly indicates that those two drugs cannot bind to the intact polymers significantly. In contrast, the uptake of radioactivity for desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone-treated pellets were markedly inhibited as compared to that of the control pellet. A small increase in the uptake of radioactivity for the 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone-treated sample is probably due to the reversible binding property of this drug to tubulin (Ray *et al.*, 1981) so that some bound drug is released during the chasing period and [ $^3\text{H}$ ]-



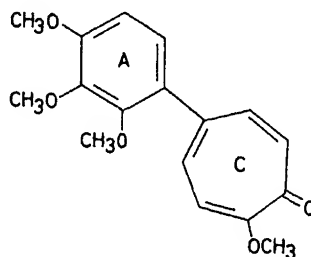


**Figure 6.** Binding of colchicine and its analogues to intact Zn(II)-induced polymers of tubulin. Aliquots of DEAE-tubulin (1.2 mg/ml) in buffer A were assembled at 37°C for 30 min in the presence of  $1 \times 10^{-4}$  M  $\text{ZnSO}_4$ . Assembled mixtures were subsequently incubated with  $1 \times 10^{-4}$  M unlabelled drugs at 37°C for 15 min. Polymers were pelleted by warm (25°C) centrifugation at 120,000 *g* for 30 min. Pellets were then resuspended, depolymerized with  $1 \times 10^{-3}$  M EDTA and subsequently incubated with  $[^3\text{H}]$ -colchicine ( $1 \times 10^{-6}$  M) at 37°C. The kinetics of uptake of  $[^3\text{H}]$ -colchicine was followed in each case by withdrawing suitable aliquots from the reaction mixture at different intervals and measuring the bound  $[^3\text{H}]$ -colchicine by DE-81 filter binding assay as described under 'materials and methods'.

(1), Control pellet obtained in the absence of any drug; (2), pellet obtained in the presence of colcemid; (3), pellet obtained in the presence of colchicine; (4), pellet obtained in the presence of 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone; (5), pellet obtained in the presence of desacetamido colchicine.



R = H, Desacetamido colchicine  
R. NH.CO.CH<sub>3</sub>, colchicine



2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone

**Figure 7.** Structure of colchicine and its analogues.

colchicine gets bound to those vacant sites. These results clearly indicate that unlike colchicine and colcemid (both of which have a B-ring substituent), desacetamido colchicine (devoid of B-ring substituent), and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (devoid of B-ring substituent) do not bind to the vacant sites of the tubulin polymer.

Although copolymerization of CD complex and tubulin dimer has been reported in normal microtubule assembly (Sternlicht and Ringel, 1979), the ratio of CD tubulin dimer in those microtubules is very low. It has been reported from our laboratory (Banerjee *et al.*, 1982) that CD complexes could also be polymerized in the presence of  $\text{ZnSO}_4$ . The incorporation of colchicine into the polymer structure has been confirmed using  $[^3\text{H}]$ -colchicine. From this behaviour of colchicine, it is conceivable that the tubulin-tubulin interaction sites involved in the process of  $\text{Zn(II)}$ -induced assembly are different from the colchicine-binding site of tubulin. Thus, from the ability of colchicine-tubulin complex to form sheets in the presence of  $\text{ZnSO}_4$  one might expect that the drug binding sites on tubulin subunits are exposed on the wall of  $\text{Zn(II)}$ -tubulin sheets. In order to test this possibility, the colchicine binding properties of the preformed  $\text{Zn(II)}$ -tubulin sheets have been studied. The results shown in figure 2 clearly indicate that the colchicine binding activity of tubulin is lost upon assembly in the presence of  $\text{Zn(II)}$ . That the loss in the drug binding activity is not due to the inactivation or poisoning of tubulin is confirmed by the fact that preincubation of tubulin with  $\text{Ca(II)}$  or vinblastine sulphate or treatment of sheets with  $\text{Zn(II)}$ -chelators (to depolymerize them) could restore the drug binding activity. Moreover, the inhibition of the drug binding activity was not observed when tubulin was incubated with  $\text{Zn(II)}$  at a protein concentration far below the critical concentration for assembly in the presence of  $\text{Zn(II)}$ . The above results indicate that the  $\text{Zn(II)}$ -tubulin sheets bind colchicine very poorly.

The assembly of tubulin at different  $\text{Zn(II)}$  concentrations was studied and it was found that inhibition of drug binding activity by  $\text{Zn(II)}$  was exhibited only when assembly was induced.  $\text{Zn(II)}$  at  $1 \times 10^{-5}$  neither induced assembly (figure 3) nor inhibited the colchicine binding activity of tubulin (figure 2). Moreover, the data obtained by the colchicine binding study correlated well with that obtained by the analysis of pellets after warm centrifugation (table 1).

Although the  $\text{Zn(II)}$ -induced assembly and normal microtubule assembly differ strikingly with respect to their sensitivity to colchicine, both polymers resemble each other with respect to colchicine binding properties *i.e.*, neither of them can bind colchicine. Thus, the assurance of accommodating a drug molecule into a polymer structure does not necessarily mean that the polymer will bind the drug. One possible explanation for the inability of  $\text{Zn(II)}$ -induced polymers to bind colchicine might be that the alteration in conformation of the tubulin molecule which is required for colchicine binding (Garland, 1978; Lambier and Engelborgh, 1981) is hindered in the assembled state as compared to the free state. Infact, this hindrance is not unlikely for tubulin in the assembled state where each tubulin is anchored by adjacent tubulin molecules. An alternative mechanism might be that although the tubulin-tubulin interaction site is different from the colchicine binding site (at least in the case of  $\text{Zn(II)}$ -induced polymers), however, it is possible that in the assembled state, the colchicine binding site is partially covered (sterically crowded) by the adjacent tubulin molecule and thus prevents a good initial contact between the colchicine and the colchicine binding site of the tubulin molecule. To test these possibilities we have chosen the B-ring analogues of colchicine *viz.*, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone which bind to tubulin at the same

clearly indicates that unlike colchicine, the binding of desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone was almost the same in the case of tubulin either in the polymerized or in the unpolymerized state. Similar results have also been obtained in the chasing experiment (figure 6). Thus, it appeared from this study that the A- and C-ring (of colchicine) binding domains on tubulin are exposed in the Zn(II)-induced polymers. Even the compound with A-, C-, and B-ring (desacetamido colchicine) could bind to tubulin in the assembled state whereas the presence of  $-NHCOCH_3$  in the B-ring makes the compound inactive. Our experiments, however, do not distinguish whether this is purely a steric effect or due to a forbidden conformational change (as needed for colchicine binding) of tubulin in the assembled state. By what means the substituents on the B-ring of colchicine alter its binding properties with normal microtubules remain to be established.

## Acknowledgements

We are indebted to Dr. Richard F. Luduena of the University of Texas Health Science Center at San Antonio, Texas, USA for helping us in doing electron microscopy and also for his valuable suggestions in preparing the manuscript. The work was supported by Council for Scientific and Industrial Research, New Delhi.

## References

- Andreu, J. M. and Timasheff, S. N. (1982a) *Biochemistry*, **21**, 6465.  
 Andreu, J. M. and Timasheff, S. N. (1982b) *Proc. Natl. Acad. Sci. USA*, **79**, 6753.  
 Banerjee, A. (1981) *Biochemical Characterization of Tubulin—Microtubule System*, Ph.D. Thesis, University of Calcutta, Calcutta.  
 Banerjee, A., Roychowdhury, S. and Bhattacharyya, B. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 1503.  
 Bhattacharyya, B. and Wolff, J. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 2627.  
 Detrich, H. W., Williams, R. C. and Wilson, L. (1982) *Biochemistry*, **21**, 2392.  
 Dustin, P. (1978) *Microtubules* (Berlin, Heidelberg, New York: Springer Verlag).  
 Garland, D. L. (1978) *Biochemistry*, **17**, 4266.  
 Gaskin, F. and Kress, Y. (1977) *J. Biol. Chem.*, **252**, 6918.  
 Haskins, K. M., Zombola, R. R., Boling, J. M., Lee, Y. C. and Himes, R. H. (1980) *Biochem. Biophys. Res. Commun.*, **95**, 1703.  
 Johnson, K. A. and Borisy, G. G. (1979) *J. Mol. Biol.*, **133**, 199.  
 Laemmli, U. K. (1970) *Nature (London)* **227**, 680.  
 Lambeir, A. and Engelborghs, Y. (1981) *J. Biol. Chem.*, **256**, 3279.  
 Larsson, H., Wallin, M. and Edstrom, A. (1976) *Exp. Cell Res.*, **100**, 104.  
 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randal, R. J. (1951) *J. Biol. Chem.*, **193**, 265.  
 Margolis, R. L. and Wilson, L. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3466.  
 Olmsted, J. B. and Borisy, G. G. (1973) *Biochemistry*, **12**, 4282.  
 Ray, K., Bhattacharyya, B. and Biswas, B. B. (1981) *J. Biol. Chem.*, **256**, 6241.  
 Saltarelli, D. and Pantaloni, D. (1982) *Biochemistry*, **21**, 2996.  
 Sandoval, I. V. and Weber, K. (1979) *J. Mol. Biol.*, **134**, 159.  
 Sternlicht, H. and Ringel, I. (1979) *J. Biol. Chem.*, **254**, 10540.  
 Ventilla, M., Cantor, C. R. and Shelanski, M. (1972) *Biochemistry*, **11**, 1554.  
 Wallin, M., Larsson, H. and Edstrom, A. (1977) *Exp. Cell Res.*, **107**, 219.  
 Weisenberg, R. C., Borisy, G. G. and Taylor, E. W. (1968) *Biochemistry*, **7**, 446.  
 Williams, J. A. and Wolff, J. (1972) *J. Cell Biol.*, **257**, 157.  
 Wilson, L. and Bryan, J. (1974) *Adv. Cell Mol. Biol.*, **3**, 21.  
 Wilson, L. and Meza, I. (1973) *J. Cell Biol.* **58**, 709.

# Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to *Vicia faba* lectin: Fluorescence-quenching studies

PRADIP K. DATTA, PRANAB S. BASU and TAPASH K. DATTA

Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Calcutta 700 032, India

**Abstract.** On binding to *Vicia faba* lectin, the fluorescence of 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside was quantitatively quenched showing that the interaction of 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside took place in a binding environment. The binding of the fluorescent sugar was saccharide specific as evidenced by the reversal of 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside fluorescence quenching by D-fructose. The association constant,  $K_a$ , values for the 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside were determined by competition study employing reversal of fluorescence quenching of 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside by D-fructose. The  $K_a$  value obtained for D-fructose was  $1.07 \pm 0.03 \times 10^4 \text{ M}^{-1}$  and for 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside was  $1.60 \pm 0.05 \times 10^4 \text{ M}^{-1}$  at  $15^\circ\text{C}$ . The  $K_a$  values of  $2.51 \pm 0.06 \times 10^4 \text{ M}^{-1}$ ,  $1.26 \pm 0.02 \times 10^4 \text{ M}^{-1}$  and  $0.56 \pm 0.01 \times 10^4 \text{ M}^{-1}$ , respectively at  $10^\circ$ ,  $20^\circ$  and  $30^\circ\text{C}$  were obtained from the Chipman equation. The relative fluorescence quenching,  $\Delta F_\infty$ , at infinite concentration of the free saccharide sites of *Vicia faba* lectin [ $P'$ ] was 93.5% at  $30^\circ\text{C}$  and the binding constant for 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside lectin interaction as derived by Yank and Hanaguchi equation was  $0.63 \pm 0.01 \times 10^4 \text{ M}^{-1}$ .

**Keywords.** *Vicia faba* lectin; lectin-saccharide binding; fluorescence quenching; binding constants.

## Introduction

Sugars, such as methyl-umbelliferyl glycosides, have been used as probes for the investigation of binding of lectins to their specific sugars. The fluorescence of methyl-umbelliferyl glycosides was considerably quenched upon binding to lectin (Dean and Homer, 1973; Privat *et al.*, 1974; Loontjens *et al.*, 1977; van Landschoot *et al.*, 1977; De Boeck *et al.*, 1981; Thompson and Lakowicz, 1984). This has provided a sensitive determination of association constants and other binding characteristics for the lectin.

Lectin from *Vicia faba* seed had been isolated and carbohydrate specificity has been reported (Goldstein and Hayes, 1978; Debray *et al.*, 1981). The structural requirements for binding of oligosaccharides and glycopeptides to immobilized *V. faba* agglutinin were investigated (Katagiri *et al.*, 1984). The spectral properties of 4-methyl- $\alpha$ -D-umbelliferyl glycosides were investigated in order to assess their usefulness as probes of microenvironment of sugar binding sites on lectin molecules (Monique *et al.*, 1984). The present communication deals with the physico-chemical aspects of 4-methyl-umbelliferyl- $\alpha$ -D-galactopyranoside (4-Met-umb-Glu)-*V. faba* lectin interaction.

4-Met-umb-Glu, 4-methyl umbelliferyl- $\alpha$ -D-galactopyranoside (4-Met-umb-Gal) and D-fructose were from Sigma Chemical Co., St. Louis, Missouri, USA.

### Sugars

The umbelliferyl sugar solutions were analysed according to Loontjens *et al.* (1977) and were found to be free from 7-hydroxy-4-methyl coumarin. The 4-Met-umb-Glu and 4-Met-umb-Gal concentrations were determined at 318 nm using  $E = 1.36 \times 10^{-4} \text{ M}^{-1} \text{ cm}^{-1}$ .

### Lectins

*V. faba* lectin was purified (Datta *et al.*, 1984a) and was dissolved in 0.15 M phosphate buffered saline pH 7.2 containing 0.1 mM  $\text{CaCl}_2$ ,  $\text{MnCl}_2$  and  $\text{MgCl}_2$ . Protein concentrations were determined using  $E_{\text{cm}}^{1\%} = 7.22$  at 280 nm (Datta *et al.*, 1984a).

### Fluorescence titrations

The binding of 4-Met-umb-Glu to *V. faba* lectin was monitored by fluorescence quenching in Aminco-Bowman Spectrophotofluorimeter, sensitivity upto  $10^{-4}$ . The excitation was done at 318 nm, and the emission spectra were recorded above 330 nm. Fluorescence quenching studies were done by titrating a definite concentration of lectin solution (225  $\mu\text{M}$ ) against varying amounts (0–200  $\mu\text{l}$ ) of 4  $\mu\text{M}$  4-Met-umb-Glu solution at 20°C. Competitive binding study with D-fructose was carried out as follows. Lectin was preincubated with 4-Met-umb-Glu (4  $\mu\text{M}$ ) and then titrated with aliquots of 0.1 M D-fructose solution at 15°C. The binding of the competitive sugar was deduced by observing the increase in fluorescence resulting from the dissociation of 4-Met-umb-Glu-*V. faba* lectin complex. The binding studies were done by titrating a fixed concentration of lectin (200  $\mu\text{M}$ ) against varying concentrations, 20–100  $\mu\text{M}$ , of 4-Met-umb-Glu at 10°, 20° and 30°C. The resulting fluorescence in the presence and absence of lectin gave the amount of bound and total glucoside respectively. Corrections were made for inner filter effect (Martens and Kagi, 1979).

## Results and discussion

The fluorescence of the sugar was completely quenched on binding to the *V. faba* lectin. It indicated that there was a change in the environment of the umbelliferyl moiety when 4-Met-umb-Glu specifically bound to *V. faba* lectin. The decrease in fluorescence was probably due to methyl-umbelliferyl group anchorage at or near the binding region through carbohydrate specific binding suggesting great reduction in the polarity of the fluorophor environment. The binding of a 4-methylumbelliferyl glycoside to a specific protein leads to a total fluorescence quenching for wheat germ agglutinin (Privat *et al.* 1974; van Landschoot *et al.* 1977) and for Con A (Dean

The index of homogeneity or heterogeneity of lectin-sugar binding was determined by Sips (1948) equation. The index of heterogeneity is 'a' and it ranges from 0 to 1. The value of  $a=1$  indicates a homogenous protein. The value of 'a' was calculated as  $1.0007 \pm 0.0003$  from the slope in figure 1 which indicated the homogenous binding between *V. faba* lectin and 4-Met-umb-Glu.

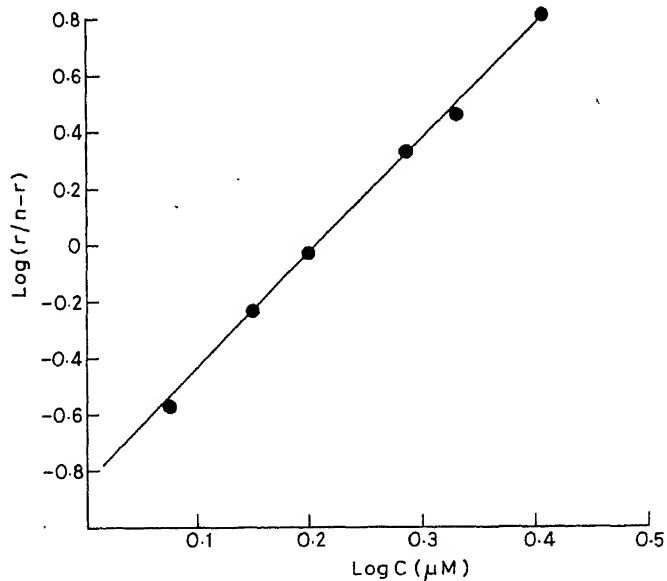
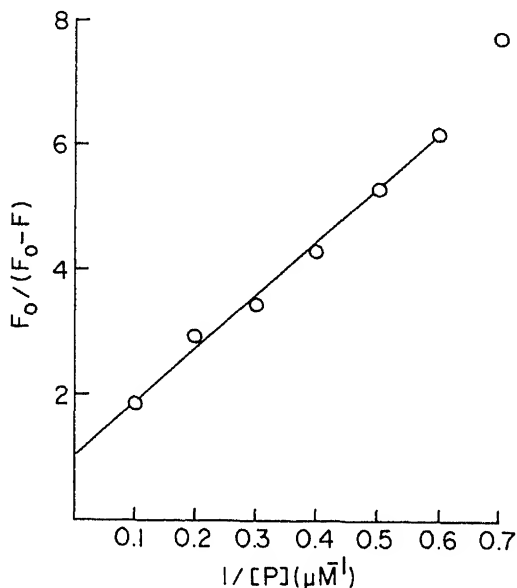


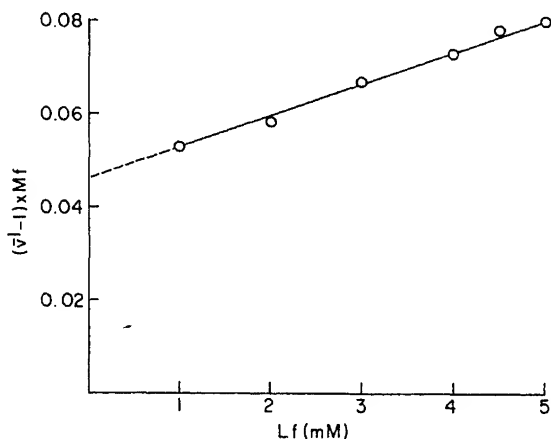
Figure 1. The index of homogeneity or heterogeneity of the purified *V. faba* lectin. Procedural details are described in the text.

The fluorescence quantum yield of 4-Met-umb-Glu bound to *V. faba* lectin was obtained by extrapolating the plot of  $F_0/(F_0 - F)$  vs  $1/[P]$  to  $1/[P]=0$  of the equation given by Dean and Horner (1973) where  $F_0$  and  $F$  were the measured fluorescence of sugar alone and in the presence of lectin respectively at protein concentration  $[P]$ . The plot gave straight line with an intercept on the ordinate  $F_0/(F_0 - F)=0.97$ , demonstrating that the fluorescence quantum yield of 4-Met-umb-Glu bound to *V. faba* lectin was zero (figure 2), and indicating that the fluorescence quantum yield could be used as a measure of ligand binding to *V. faba* lectin in titration. The number of sugar binding sites of *V. faba* lectin ( $n=4$ ) was reported earlier (Datta *et al.*, 1984b; Datta *et al.*, 1984c; Datta *et al.*, 1986).

The quenching of 4-Met-umb-Glu on binding to *V. faba* lectin was completely reversed by addition of 0.1 M D-fructose, showing that quenching was due to sugar specific binding (figure 3). The fluorescence of 4-Met-umb-Gal was not quenched when titrated with *V. faba* lectin, thus excluding the presence of any other binding site with which 4-methyl umbelliferyl moiety of the galactoside could interact. The lectin pretreated with D-fructose did not quench the fluorescence of 4-Met-umb-Glu and demonstrated that both the sugars competed for the same binding site of the *V. faba* lectin. The binding of D-fructose to the *V. faba* lectin ( $P$ ) in the presence of 4-



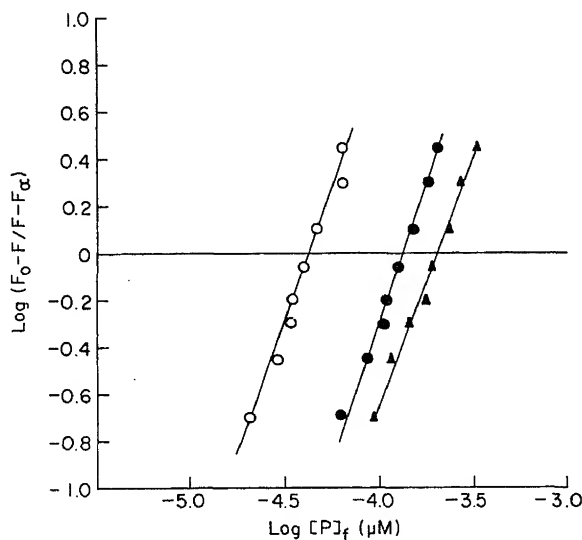
**Figure 2.** Plot of the fluorescence intensity changes ( $F_0/\Delta F$ ) of Met-umb-Glu upon addition of *V. faba* lectin at 20°C. Aliquots of a solution of *V. faba* lectin, 225  $\mu M$ , were added to 1 ml of Met-umb-Glu (4  $\mu M$ ) solution. The experimental procedure is given in the text.



**Figure 3.** Competitive binding of saccharides with *V. faba* lectin at 15°C.  $[L]_f$  is the free concentration of the competing sugar (fructose);  $[M]_f$  is the free concentration of Met-umb-Glu. The fraction of  $M$  bound per total protein,  $[PM]/[P]_t$ , is represented by  $v$ . *V. faba* lectin 160  $\mu M$ , was incubated with Met-umb-Glu (4  $\mu M$ ) and then titrated with aliquots of 0.1 M D-fructose solution. The rest of the procedure is described in the text.

The association constant  $K_a$  for the competing sugar ligand could be determined from the slope and intercept of the line obtained from a straight line which was

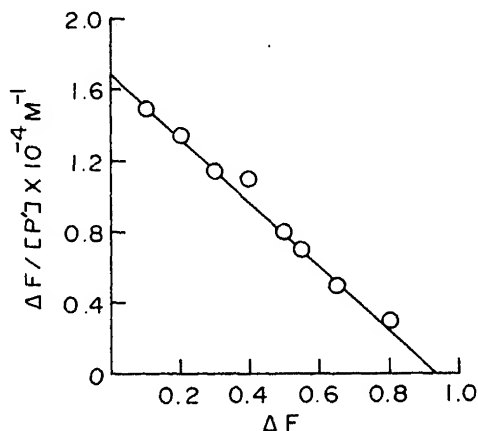
The values of association constant  $K_a$  of the lectin-sugar interaction was estimated using the quenching values of 4-Met-umb-Glu fluorescence by varying concentrations of *V. faba* lectin. A plot of  $\log (F_0 - F)/(F - F_\infty)$  against  $\log [P]_f$  from Chipman (1967) equation gave a straight line where  $F_0$ ,  $F$  and  $F_\infty$  were the values of the fluorescence maxima of 4-Met-umb-Glu in the absence of protein, in the presence of protein and at infinite protein concentration (figure 4) respectively and  $[P]_f$  was the free protein concentration. The values of the association constants  $2.52 \pm 0.06 \times 10^4 \text{ M}^{-1}$  at  $10^\circ\text{C}$ ,  $1.26 \pm 0.02 \times 10^4 \text{ M}^{-1}$  at  $20^\circ\text{C}$  and  $0.56 \pm 0.01 \times 10^4 \text{ M}^{-1}$  at  $30^\circ\text{C}$  were obtained from the intercepts on the abscissa.



**Figure 4.** A quenching of the fluorescence spectra of Met-umb-Glu by *V. faba* lectin. The values of the association constants were obtained from the intercept on the x-axis. (○),  $10^\circ\text{C}$ ; (●),  $20^\circ\text{C}$  and (▲),  $30^\circ\text{C}$ . The experimental details are given in the text.

The association constant  $K_a$  was determined at  $30^\circ\text{C}$  in another set of experiment by the method of Yank and Hamaguchi (1980) as modified by De Boeck *et al.* (1981) (figure 5). The association constant  $0.63 \pm 0.01 \times 10^4 \text{ M}^{-1}$  at  $30^\circ\text{C}$  was calculated from the slope of a straight line obtained by plotting  $\Delta F/[P']$  vs  $\Delta F$  where  $\Delta F$  is the relative fluorescence quenching and  $[P']$  was the concentration of the free saccharide sites of *V. faba* lectin.  $\Delta F_\infty$  at infinite  $[P']$  was 93.5% as calculated from the intercept. The fluorescence of 4-Met-umb-Glu was quantitatively quenched on binding to *V. faba* lectin. The binding of the fluorescent sugar was saccharide specific as evidenced by the reversal of 4-Met-umb-Glu fluorescence quenching by D-fructose. The plot of fluorescence quantum yield of 4-Met-umb-Glu bound to *V. faba* lectin was a sensitive measurement of the equilibrium parameters at different temperatures.





**Figure 5.** Association constant of purified *V. juba* lectin from  $\Delta F/[P']$  vs  $\Delta F$  plot.

$\Delta F$  is the relative fluorescence quenching and  $[P']$  is the concentration of free sites; both are corrected for dilution. The values of  $K$  (in  $M^{-1}$ ) and  $\Delta F_{\infty}$  at infinite  $[P']$  were calculated from the slope, and intercept in the ordinate respectively.

## Acknowledgement

One of the authors (P. K. D.) is a Research Associate, IICB-CSIR.

## References

- Bessler, W., Shafer, J. A. and Goldstein, I. J. (1974) *J. Biol. Chem.*, **249**, 2819.  
 de Boeck, H., Loontjens, F. G., Delmotte, F. M. and de Bruyne, C. K. (1981) *FEBS Lett.*, **126**, 227.  
 Chipman, D. M., Grisaro, U. and Sharon, N. (1967) *J. Biol. Chem.*, **242**, 4388.  
 Datta, P. K., Basu, P. S. and Datta, T. K. (1984a) *Prep. Biochem.*, **14**, 373.  
 Datta, P. K., Basu, P. S. and Datta, T. K. (1984b) *IRCS Med. Sci.*, **12**, 687.  
 Datta, P. K., Basu, P. S. and Datta, T. K. (1984c) *Indian J. Biochem. Biophys.*, **21**, 174.  
 Datta, P. K., Basu, P. S. and Datta, T. K. (1986) *Carbohydr. Res.*, **154**, 259.  
 Dean, B. R. and Homer, R. B. (1973) *Biochim. Biophys. Acta*, **322**, 141.  
 Debray, H., Decout, D., Strecher, G., Spik, G. and Montreuil, J. (1981) *Eur. J. Biochem.*, **117**, 41.  
 Goldstein, I. J. and Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.*, **35**, 128.  
 Katagiri, Y., Yamamoto, K., Tsuji, T., Osawa, T. (1984) *Carbohydr. Res.*, **129**, 257.  
 Loontjens, F.-G., Clegg, R. M. and Jovin, T. M. (1977) *Biochemistry*, **16**, 159.  
 Mertens, M. L. and Kagi, J. H. R. (1979) *Anal. Biochem.*, **96**, 448.  
 Monique, D., Michel, V., Khushi, M. L. and Jean-Pierre, F. (1984) *Arch. Biochem. Biophys.*, **232**, 640.  
 Privat, J. P., Delmotte, F. and Monsigny, M. (1974) *FEBS Lett.*, **46**, 229.  
 Sips, R. (1948) *J. Chem. Phys.*, **16**, 490.  
 Thompson, R. B. and Lakowicz, J. R. (1984) *Biochim. Biophys. Acta*, **790**, 87.  
 van Landschoot, A., Loontjens, F. G., Clegg, R. M., Sharon, N. and DeBruyne, C. K. (1977) *Eur. J. Biochem.*, **79**, 275.  
 Yank, Y. and Hamaguchi, K. (1980) *J. Biochem. (Tokyo)*, **88**, 829.

# Abnormal erythrocyte membrane phospholipid organisation in chronic myeloid leukaemia

A. KUMAR, S. DANIEL\*, S. S. AGARWAL\* and C. M. GUPTA†

Division of Biochemistry, Central Drug Research Institute, Lucknow 226 001, India

\*Department of Medicine, King George's Medical College, Lucknow 226 003, India

**Abstract.** The membrane phospholipid organisation in the red cells of humans suffering from chronic myeloid leukaemia has been analysed using the amino-group labelling reagent trinitrobenzenesulphonic acid and the fluid-sensing fluorophore, Merocyanine 540. Unlike the normal human erythrocytes, trinitrobenzenesulphonic acid in intact chronic myeloid leukaemia erythrocytes modified about 30% phosphatidylserine, under controlled conditions. Also, the chronic myeloid leukaemia red cells, but not the normal cells, were found to bind the fluorescent dye Merocyanine 540. These results demonstrate that loss of the transmembrane phospholipid asymmetry in chronic myeloid leukaemia erythrocytes is accompanied by an enhancement in the outer surface fluidity and, therefore, suggest that the red cells membrane phase-state asymmetry originates probably from the asymmetric arrangements of phospholipids across the membrane bilayer.

**Keywords.** Phospholipid asymmetry; erythrocytes; phase-state asymmetry; chronic myeloid leukaemia.

## Introduction

Chronic myeloid leukaemia (CML) is a clonal disorder common to granulocyte, platelet and erythrocyte precursors (Champlin and Golde, 1985; Gale and Cannani, 1985). Our recent studies have shown that the CML erythrocyte spectrin becomes abnormal due to crosslinking of its two subunits *via* disulphide bonds (Kumar and Gupta, 1983). This abnormality in these cells has been found to be associated with the presence of substantial amounts of Phosphatidylserine (PS) in the outer surface of the membrane bilayer (Kumar and Gupta, 1983). We now report that the amino-group labelling reagent, trinitrobenzenesulphonic acid (TNBS); can readily modify about 30% PS in the intact CML erythrocytes. In addition, we show that the fluid-sensing probe, Merocyanine 540 (Mc 540), binds the CML cells but not the normal erythrocytes. These results indicate that the membrane skeletal defects in CML erythrocytes are associated not only with loss of the transmembrane phospholipid asymmetry but also lead to an increase in the outer monolayer fluidity.

## Materials and methods

All the chemicals and reagents used in this study were of the highest purity available. TNBS, bovine serum albumin (fatty acid free) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA.

†To whom all correspondence should be addressed.

Abbreviations used: CML, Chronic myeloid leukaemia; CML erythrocytes, red cells of humans afflicted with CML; PS, phosphatidylserine; TNBS, trinitrobenzenesulphonic acid; Mc, Merocyanine 540; DTNB,

Mc 540 was a gift from Dr. Rolf D. Walter, Bernhard Nocht Institute, Hamburg, Federal Republic of Germany. Fetal calf serum was from Difco, USA.

Blood from leukaemic male patients (aged 35–50 years) and from healthy adult donors was obtained from King George's Medical College, Lucknow, and drawn by venepuncture into heparinized glass tubes. All the patients studied here were established cases of CML as shown by their haematological and clinical analysis. Erythrocytes from whole blood were isolated as described earlier (Kumar and Gupta, 1983).

### *Labelling of erythrocytes with TNBS*

Erythrocytes were labelled with TNBS essentially according to the method of Gordeski *et al.* (1975). Incubations were done for varying periods of time (3, 6 and 9 h) at 10°, 20° and 30°C. At 20°C, incubations were carried out both in the presence and absence of an anion channel protein inhibitor, DTNB (Reithmeier, 1983; Toon *et al.*, 1985). Cells from incubation mixtures were harvested by centrifugation and washed several times with saline (pH 7.4). Lipids from washed cells were extracted and chromatographed on silica gel G-60 thin-layer chromatography plates according to the published procedures (Kumar and Gupta, 1983) and the percentage of TNBS labelling was determined as described earlier (Gupta and Mishra, 1981).

### *Binding of erythrocytes with Mc 540*

Studies on binding of Mc 540 to both normal human and CML erythrocytes were carried out according to Schlegel *et al.* (1980). The optimum conditions of dye concentrations, duration of incubation and serum concentration were established so that no fluorescence was observed in the normal cells due to non-specific adherence of the dye. Using these conditions, the binding of Mc 540 to CML erythrocytes was carried out and compared. Fluorescence microscopy was performed with a Zeiss microscope using epillumination with green excitation filter BP 546/12 and barrier filter LP 590. Erythrocytes were photographed using ASA 400 Kodak VR film.

## **Results**

### *Erythrocyte aminophospholipid labelling with TNBS*

The amino-group labelling reagent, TNBS, has widely been used as an external membrane probe to determine the transbilayer amino-phospholipid distributions in the membranes of various types of cells (reviewed by Etemadi, 1980; Roelofsens, 1982). However, conditions need to be optimised for using this reagent in the studies of phospholipid distribution across the erythrocyte membrane, as it is known that TNBS penetrates into the human red cells upon prolonged incubations and/or at temperatures > 20°C (Gordeski *et al.*, 1975; Haest *et al.*, 1981). Therefore, normal as well as CML erythrocytes were treated with the reagent at various temperatures for

**Table 1.** Labelling of erythrocytes with TNBS.

Sample	Incubation temperature (°C)	Incubation time (h)	Labelling of aminophospholipids	
			PE (%)	PS (%)
Normal erythrocytes	10	9	16.4 ± 1.6	0
	20	3	22.4 ± 2.9	0
		6	24.1 ± 3.2	0
		9	25.3 ± 3.1	0
	30	3	24.2 ± 2.6	40.4 ± 2.0
		6	39.2 ± 3.0	45.2 ± 3.9
		9	50.8 ± 1.6	54.6 ± 3.8
CML erythrocytes	10	9	27.9 ± 2.2	24.7 ± 0.8
	20	3	30.1 ± 2.6	28.7 ± 1.8
		6	35.8 ± 2.5	40.8 ± 1.9
		9	42.3 ± 1.2	44.0 ± 0.9

Values are mean of 6–8 determinations ± S.D.

°C was a trifle inhibited as the amount of phosphatidylethanolamine (PE) labelled the normal cells was less than that reported to be present in the outer monolayer (Ordovsky *et al.*, 1975). However, raising the incubation temperature to 30°C resulted in complete lysis of CML erythrocytes and in labelling of both PE and PS in the normal cells. Moreover, even at 20°C the amounts of labelled aminophospholipids increased significantly with time in case of CML erythrocytes. These results indicate that TNBS readily penetrates into both normal and CML erythrocytes at 20°C and in the CML cells only upon prolonged incubation at 20°C. The labelling at 30°C of CML cells could not be continued to saturation as incubation of these cells beyond 9 h, resulted in heavy lysis.

Penetration of TNBS into red cells is known to be inhibited upon blocking the red cell anion transfer system by an appropriate anion channel protein inhibitor (Haest *et al.*, 1981). Therefore, we treated the red cells with TNBS at 20°C in the presence of an anion channel protein inhibitor, DTNB (Reithmeier, 1983; Toon *et al.*, 1985). Table 2 shows that under these conditions about 19% PE was modified in the normal cells whereas in CML erythrocytes, about 26% PE and 30% PS were labelled. These amounts of labelled amino-phospholipids are comparable to those

**Table 2.** Labelling of erythrocytes with TNBS in the presence of DTNB.

Sample	DTNB (mM)	Labelling of aminophospholipids	
		PE (%)	PS (%)
Normal erythrocytes	5	18.8 ± 0.3	0
	10	19.1 ± 0.6	0
CML erythrocytes	5	26.3 ± 0.5	29.1 ± 0.2
	10	25.6 ± 2.0	33.6 ± 3.4

Incubation were done at 20°C for 9 h

labelled by TNBS at 10°C or 20°C (3 h) in the absence of DTNB. From these findings, it may be concluded that the typical transmembrane phospholipid asymmetry in erythrocytes (Schwartz *et al.*, 1985) is lost during CML. This is quite consistent with the earlier studies (Kumar and Gupta, 1983).

#### *Mc 540 binding to erythrocytes*

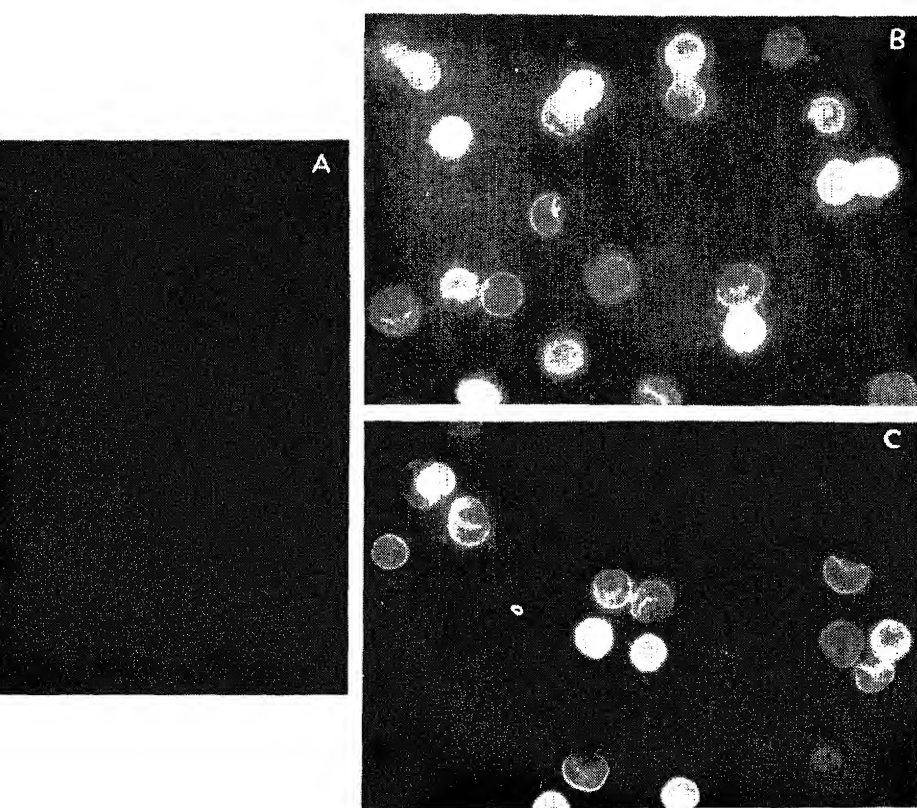
Mc 540 is a negatively charged fluorescent dye, having the unique characteristic feature to show enhanced fluorescence on intercalation with the hydrophobic domain of the bilayer, and it binds preferentially to relatively disordered or fluid domains in the outer leaflet of the membrane bilayer in intact cells (Williamson *et al.*, 1982). The dye has a low affinity for the normal red cell membrane, and even this affinity is abolished by the addition of a competing serum at a concentration of 5%. For this purpose, autologous plasma, AB serum, fetal calf serum or even bovine serum albumin can be used. Of the various sera tested, AB serum was found to give the best results. The optimum results were obtained by using 5% AB serum and a dye concentration of 20 µg/ml cell suspension. Incubation of  $10^5$ – $10^6$  red cells/ml for 10 min at 37°C was adequate. Under these conditions, 40–60% CML erythrocytes were stained with the dye whereas the normal red cells completely failed to fluoresce after the Mc 540 treatment (figure 1). These results strongly indicate that fluidity of the outer leaflet of the erythrocyte membrane bilayer is enhanced in CML.

#### **Discussion**

Erythrocyte membrane phospholipids are asymmetrically distributed across the membrane bilayer (reviewed by Schwartz *et al.*, 1985). Phosphatidylcholine and sphingomyelin are localized mainly in the outer monolayer whereas PE and PS are present almost exclusively in the inner monolayer. This typical transmembrane phospholipid asymmetry was absent in CML erythrocytes, since considerably larger amounts of aminophospholipids were found to be located in the external monolayer of these cells, as compared with the normal human erythrocytes. In the normal cells, about 19% PE and 0% PS were labelled by TNBS whereas in the CML cells, this reagent modified about 26% PE and 30% PS. These amounts of the labelled aminophospholipids should represent the external phospholipids, as similar amounts of these lipids have earlier been shown to be accessible to phospholipase A<sub>2</sub> in the intact CML erythrocytes (Kumar and Gupta, 1983).

Human red cell membrane choline-phospholipids are known to be more saturated than the aminophospholipids (Williams *et al.*, 1966). Therefore, movements of PE and PS from the inner to the outer monolayer should lead to an increase in the outer surface fluidity. This is quite consistent with the present observation that Mc 540 readily binds to the CML erythrocytes but fails to stain the normal red cells.

This study demonstrates that during CML loss of the transmembrane phospholipid asymmetry in erythrocytes is associated with an increase in the outer monolayer fluidity. These membrane changes in the cells are probably induced by the structural defects in the membrane skeletal proteins (Kumar and Gupta, 1983), as associations of these proteins with the membrane bilayer seem to stabilize the membrane phos-



**Figure 1.** Staining of human erythrocytes with Mc 540. All the cells were photographed at the same magnification and exposure time of 30 s. A, Normal erythrocytes; B and C, CML erythrocytes. Note that the membranes of CML erythrocytes fluoresce after Mc 540 staining but under identical conditions, the normal red cells do not show any fluorescence.

Earlier studies which showed that differential fluidities across the erythrocyte membrane are due to asymmetric transbilayer distributions of the membrane phospholipids (Williamson *et al.*, 1982).

Further, it has earlier been suggested that alterations in the membrane phospholipid asymmetry leads to several pathological disorders (Schwartz *et al.*, 1985). Severe anaemia in CML patients could be due to the externalisation of PS, as it would accelerate the destruction of the CML red cells by the macrophages (Schroit *et al.*, 1985).

## References

- Amplin, R. E. and Golde, D. W. (1985) *Blood*, **65**, 1039.
- Amadi, A.-H. (1980) *Biochim. Biophys. Acta*, **604**, 423.
- Amadi, R. P. and Cannani, E. (1985) *Br. J. Haematol.*, **60**, 395.
- Ardesky, S. E., Marinetti, G. V. and Love, R. (1975) *J. Membr. Biol.*, **20**, 111.

- Haest, C. W. M. (1982) *Biochim. Biophys. Acta*, **694**, 331.
- Kumar, A. and Gupta, C. M. (1983) *Nature (London)*, **303**, 632.
- Reithmeier, R. A. F. (1983) *Biochim. Biophys. Acta*, **732**, 122.
- Roelofs, B. (1982) *J. Toxicol. (Toxin Rev.)*, **1**, 87.
- Schlegel, R. A., Phelps, B. M., Waggoner, A., Terada, L. and Williamson, P. (1980) *Cell*, **20**, 321.
- Schroit, A. J., Madsen, J. W. and Tanaka, Y. (1985) *J. Biol. Chem.*, **260**, 5131.
- Schwartz, R. S., Chiu, D. T.-Y. and Lubin, B. (1985) *Curr. Top. Hematol.*, **5**, 63.
- Toon, M. R., Dorogi, P. L., Lukacovic, M. L. and Solomon, A. K. (1985) *Biochim. Biophys. Acta*, **818**, 158.
- Williams, J. H., Kuchmak, M. and Witter, R. (1966) *Lipids*, **1**, 391.
- Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.-R. and Schlegel, R. A. (1982) *Cell*, **30**, 725.

# Immobilization of *Kluyvera citrophila* penicillin acylase on controlled-pore ceramics

A. M. BODHE and C. SIVARAMAN\*

Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

**Abstract.** Penicillin acylase was purified from *Kluyvera citrophila* and immobilized on glutaraldehyde derivatives of silanized controlled-pore ceramics. The behaviour of the enzyme attached to  $\text{TiO}_2$ ,  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$  in the hydrolytic reaction are compared with that of the native enzyme as well as of the enzyme bound to CNBr-activated Sepharose 4B. The enzyme immobilized on  $\text{TiO}_2$  shows an efficiency of about 95% on the basis of protein bound. The penicillin acylase attached to  $\text{SiO}_2$ , unlike the enzyme immobilized on  $\text{TiO}_2$ ,  $\text{Al}_2\text{O}_3$  and Sepharose loses activity markedly in every cycle of use.

**Keywords.** *Kluyvera citrophila*; penicillin acylase; controlled-pore ceramics; immobilization; 6-aminopenicillanic acid.

## Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11 also known as penicillin amidase), catalyses the deacylation of penicillins with the formation of 6-aminopenicillanic acid (6-APA) and the side chain. The immobilized enzyme finds industrial application in the manufacture of 6-APA required for the production of semisynthetic penicillins and several methods have been described in the literature for the attachment of the enzyme to solid supports (Abbott, 1976; Subramanian *et al.*, 1978; Park *et al.*, 1982; Savidge, 1984; Lowe, 1985). These procedures include adsorption on carriers, inter-molecular crosslinking, entrapment in gels and covalent attachment to polysaccharide carriers and to synthetic polymers.

The present paper reports the immobilization of penicillin acylase from *Kluyvera citrophila* by covalent attachment to silanized derivatives of controlled-pore ceramics of titania, alumina and silica. The properties of the enzyme attached to the inorganic carriers are compared with those of the native enzyme and of the enzyme attached covalently to Sepharose. The *K. citrophila* enzyme, like most of the other penicillin acylases of bacterial origin, preferentially hydrolyses benzylpenicillin (Shimizu *et al.*, 1975).

## Materials and methods

Benzylpenicillin, phenoxymethylpenicillin, ampicillin, 6-APA and streptomycin sulphate were obtained from Hindustan Antibiotics Ltd., Poona.

DL-Phenylglycine methyl ester was prepared by esterification of the DL-acid as described by Greenstein and Winitz (1961). Crystalline bovine serum albumin (BSA) and other marker proteins were obtained from Sigma Chemical Co., St. Louis,

\*To whom all correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; M<sub>r</sub>, molecular weight.



Missouri, USA. Sephadex G-200 and Sepharose 4B were from Pharmacia Fine Chemicals, Sweden. DEAE-cellulose was chromatography grade DE-52 from Whatman Ltd., UK.

The following controlled-pore glass and ceramics were gifts from Corning Glass Works, USA; SiO<sub>2</sub> (pore diameter 425 Å; surface area 40 m<sup>2</sup>/g, 45–80 mesh), TiO<sub>2</sub> (Lot 2511-T, pore diameter 560 Å; surface area 10 m<sup>2</sup>/g, 30–45 mesh), Al<sub>2</sub>O<sub>3</sub> (Lot 2011-A, pore diameter 225 Å; surface area 90 m<sup>2</sup>/g, 30–45 mesh). The inorganic supports were cleaned before use by boiling with 5% nitric acid for 45 min followed by cooling, washing with distilled water and drying at 115°C for 24 h.

Cyanogen bromide and glutaraldehyde were obtained from Fluka, Switzerland and  $\gamma$ -aminopropyltriethoxysilane was obtained from Pierce Chemical Co., USA.

### *Growth of organism*

*K. citrophila* ATCC 21285 was obtained from the American Type Culture Collection. The culture was maintained on nutrient agar medium and subcultured every month. For obtaining cells required for the isolation of penicillin acylase the following growth medium was used: beef extract, 10 g; Difco peptone, 10 g; NaCl, 5 g; phenylacetic acid, 2.04 g; and water to make 1 L after adjustment of pH to 7.2 with 1 N NaOH. The organism was grown in shake flasks for 48 h at 30°C and cells were harvested by centrifugation.

### *Extraction of cells*

Wet cells suspended in 0.1 M potassium phosphate buffer, pH 7.5 (10 ml/g) were cooled in an ice bath and sonicated for 2 min in the Biosonic III Sonic oscillator (Bronwill Scientific Co., USA). The cell debris was removed by centrifugation at 15000 *g* for 20 min at 4°C.

### *Purification of penicillin acylase*

The initial steps in the purification procedure were essentially similar to those used by Shimizu *et al.* (1975). All operations were carried out at 0–4°C. Batches of 100 ml each of the extract of sonicated cells were processed at a time. The extract was treated with streptomycin sulphate (1.4 g) under constant stirring and precipitated nucleic acids were removed by centrifugation at 50,000 *g* for 30 min.

The supernatant from the streptomycin step was stirred and fractionated with powdered ammonium sulphate. Penicillin acylase activity which precipitated mainly between 0.45 and 0.9 saturation was collected by centrifugation and dissolved in 0.01 M potassium phosphate buffer, pH 7.5 (17 ml) and dialyzed against 1 L of the buffer of the same concentration for 16 h with 3 changes. The dialyzed ammonium sulphate fraction was centrifuged at 10,000 *g* for 20 min and the clear supernatant

trated by ultrafiltration through a PM-10 membrane to a final volume of about 3–4 ml.

The concentrated enzyme solution was loaded on Sephadex G-200 ( $2 \times 100$  cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5 and eluted with buffer of the same composition at a flow rate of 4 ml/h and fractions of 1 ml volume were collected, and fractions containing enzyme activity were pooled and stored at  $-20^{\circ}\text{C}$ .

#### *Estimation of molecular weight*

The molecular weight ( $M_r$ ) value of the enzyme was estimated by gel filtration through Sephadex G-200 ( $2 \times 100$  cm) column standardized with catalase (250,000), BSA (68000), ovalbumin (45000), myoglobin (17500) and cytochrome *c* (13000). Subunit  $M_r$  was determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 7.5% gels (Weber and Osborn, 1969) using myoglobin, ovalbumin and BSA monomer and dimer as markers.

#### *Assay of enzyme activities*

**Soluble enzyme:** The assay for penicillin acylase activity was done essentially as described by Balasingham *et al.* (1972). The test system contained 2% w/v substrate in 0.1 M potassium phosphate buffer, pH 7.5. Enzyme activity measurements were made at  $40^{\circ}\text{C}$  by determining 6-APA formed with *p*-dimethylaminobenzaldehyde according to the procedure described by Bomstein and Evans (1965). One unit of enzyme activity is defined as the amount of enzyme catalysing the hydrolysis of  $1\ \mu\text{mol}$  of substrate in 1 min under assay condition.

$\beta$ -Lactamase activity was checked by the micro-iodometric procedure described by Ross and O'Callaghan (1975).

**Immobilized enzyme:** Preparations were assayed in stirred, water-jacketed vessels maintained at  $40^{\circ}\text{C}$ . The assay medium was similar to that used for the soluble enzyme. The final volume of the reaction mixture was 50 ml. Aliquots were withdrawn at 15 min intervals and rapidly filtered off through a sintered glass filter under suction and the 6-APA in the filtrate was assayed with *p*-dimethylaminobenzaldehyde as described earlier.

**Enzyme kinetics:** The  $K_m$  for benzylpenicillin was calculated from the double reciprocal plots by least squares analysis. Studies on the temperature dependence of activity were in the temperature range of  $25^{\circ}$ – $40^{\circ}\text{C}$ . The initial reaction rates ( $v$ ) were measured as described under assay of enzyme activity. The activation energy was calculated from  $\log_{10} v$  versus  $1/T$  plots.

#### *Protein assay*

Sephacrose 4B was activated with CNBr using 1 M  $\text{Na}_2\text{CO}_3$  for controlling the pH during activation as recommended by March *et al.* (1974).

One volume of an aqueous slurry of washed Sepharose 4B beads (1:1 w/v) was treated with 2 M  $\text{Na}_2\text{CO}_3$  (1.5 volume). An aqueous solution of CNBr (100 mg/ml; 0.5 volume) was added to the beads and the mixture agitated gently for 10 min at 18°–22°C. The mixture was then cooled with addition of crushed ice. The activated beads were washed rapidly on a sintered glass filter with ice cold 0.1 M  $\text{NaHCO}_3$  containing 0.17 M NaCl (25 volumes). Filtration and washing were completed within 10 min by applying gentle suction. The washed beads were immediately added to an ice cold solution of the purified enzyme in 0.05 M potassium phosphate buffer, pH 7.5 (0.4 mg protein/ml; 3–4 U/g wet beads). The beads were gently agitated for 16 h at 0°–4°C. The beads were then filtered off and washed with cold coupling buffer. The pH of the filtrate was approximately 8 at the end of the coupling reaction. The filtrate and washings were separately tested for activity and protein. Any unreacted, activated groups on the support were masked by treatment with 1 M ethanolamine at pH 8 at 0°–5°C for 1 h. The beads were finally filtered off, washed with 0.1 M potassium phosphate buffer, pH 7.5 (10 volumes) and stored at 0°–4°C in the same buffer.

#### *Controlled-pore glass and ceramic supports*

Enzyme was covalently bound to the surface of the inorganic carriers through silanization with  $\gamma$ -aminopropyltriethoxysilane followed by coupling of the protein with the use of glutaraldehyde according to the procedure described by Weetall (1976).

*Preparation of alkylamine carrier:* The cleaned dry carrier (1 g) was treated with 10% (w/v)  $\gamma$ -aminopropyltriethoxysilane dissolved in toluene (75 ml) and refluxed for 16 h. The mixture was then cooled and the carrier filtered off, washed thoroughly first with toluene and then with acetone and air dried.

*Preparation of glutaraldehyde derivative of alkylamine carrier:* The alkylamine carrier (1 g) was covered with 2.5% aqueous glutaraldehyde solution (10 ml) and agitated with a teflon-coated magnetic stirring bar for 1 h at room temperature. The carrier was then filtered off on a Buchner funnel and washed with distilled water to remove excess of glutaraldehyde.

*Coupling of enzyme to the aldehyde carrier:* The aldehyde derivative of the carrier (1 g) was treated with the enzyme solution (5 ml containing 5 mg protein and about 30 U activity in 0.1 M potassium phosphate buffer, pH 7.5). The mixture was stirred for 1 h at room temperature. The carrier was filtered off and washed with 0.1 M potassium phosphate buffer, pH 7.5. The filtrate and washings were pooled and assayed for enzyme activity.

The operational stability of immobilized penicillin acylase preparations was determined in repeated cycles of use at 40°C and a substrate concentration of approximately 2% benzylpenicillin K-salt taken in a 10 ml capacity water jacketed glass reaction vessel provided with a combination glass electrode probe for monitoring pH and a teflon-coated magnetic stirring bar for continuous agitation. 9 ml reaction mixture containing 200 mg benzylpenicillin K-salt was treated with an adequate amount of immobilized enzyme for complete hydrolysis of the substrate in 2–3 h. The pH was maintained at 7.5 with addition of 0.5–1.0 M aqueous ammonia solution dispensed from a microburette. The progress of reaction was monitored by assay of 6-APA in samples withdrawn periodically. The completion of hydrolysis was apparent from the cessation of proton release. After the completion of reaction, the immobilized enzyme was filtered off and washed with water till washings were free from 6-APA. Determination of 6-APA in filtrate and washings established 95–100% hydrolysis of the substrate.

Aliquots of the immobilized enzyme were assayed for enzyme activity after set cycles of use.

#### *Synthetic activity in ampicillin production from 6-APA and phenylglycine methyl ester*

Synthetic activity was followed essentially as described by Takasawa *et al.* (1972). The reaction mixture (6.5 ml) contained 6-APA, 50 mg; DL-phenylglycine methyl ester HCl, 125 mg; potassium phosphate buffer, 0.03 M, final pH adjusted to 6.5 with 1 N NaOH. This system was maintained at 40°C. Reaction was initiated with addition of the purified enzyme immobilized on controlled-pore TiO<sub>2</sub> (8 U, 250 mg) and the mixture stirred continuously. Aliquots were withdrawn hourly for assay of 6-APA using *p*-dimethylaminobenzaldehyde. Neither phenylglycine methyl ester nor ampicillin gave any colour with the reagent and their presence did not interfere in the assay. Aliquots were also taken for characterization of the product by reversed phase thin layer chromatography on silica gel (Biagi *et al.*, 1969).

## **Results**

#### *Purification of K. citrophila penicillin acylase*

The results obtained in a typical batch are summarized in table 1.

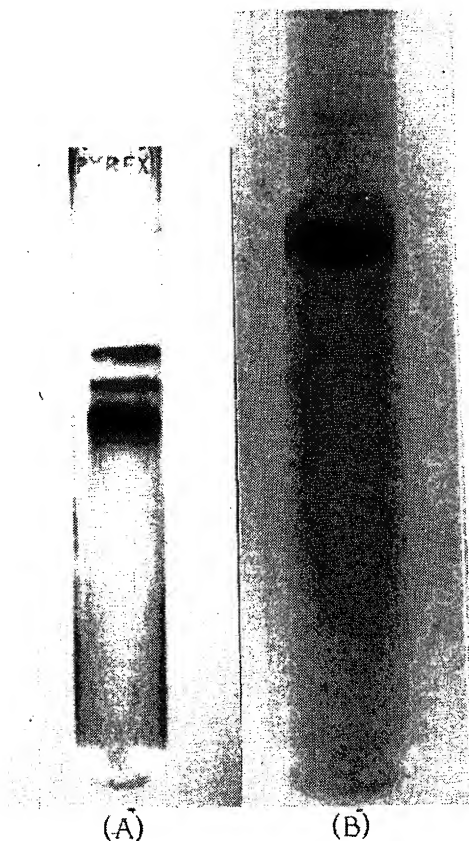
The overall purification was about 50-fold with a recovery of about 25% of initial activity. The specific activity of the final preparation towards benzylpenicillin was 8 U/mg. Shimizu *et al.* (1975) have reported a value of about 14.5 U/mg for the enzyme purified from *K. citrophila* (KY 7844).

#### *Properties of K. citrophila penicillin acylase*

Disc electrophoresis of the Sephadex fraction carried out in 7.5% polyacrylamide at pH 8.3 according to Davis (1964) showed the presence of 3 bands (figure 1A).

**Table 1.** Purification of penicillin acylase from *K. citrophila*.

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Cell free extract	100	120	750	0.16	100
Ammonium sulphate fraction (0.45–0.90 sat.) dissolved in 0.01 M potassium phosphate buffer, pH 7.5 and dialysed	42	83	453	0.18	69
DEAE-Cellulose fraction concentrated by ultrafiltration	4	52	22	2.4	43
Sephadex G-200 fraction	5	30	3.8	8.0	25



**Figure 1.** PAGE of *K. citrophila* penicillin acylase. (A), 7% polyacrylamide gel; Tris-glycine buffer, pH 8.3; 4 mA/tube; 1.5 h; 4°C; Protein loaded 100 µg; (B), SDS-polyacrylamide gel; 0.1 M sodium phosphate buffer, pH 7.3; SDS, 0.1%; 8 mA/tube; 4 h. Total protein loaded, 100 µg.

Segments cut out from the unstained gel when assayed for penicillin acylase activity showed that all the 3 components were active. SDS-PAGE showed almost exclusively a single band of *M*. 67000 (Figure 1B). Shimizu *et al.* (1975) have purified

or only a single component on isoelectric focusing. The reasons for the multiple components obtained in the present work are not clear. Such heterogeneity in penicillin acylases has been reported for the enzymes obtained from other bacterial sources. Vandamme and Voets (1975) have reported the presence of 2 active components in the enzyme purified from *Erwinia aroideae*. Kashe *et al.* (1984) have separated atleast 5 active components from the enzyme purified from *Escherichia coli*.

The  $M_r$  estimated by gel filtration was approximately 60000 which is in agreement with the reported value of  $63000 \pm 3000$  for the enzyme from this source (Shimizu *et al.*, 1975). The optimum pH for the cleavage of benzylpenicillin was 7.5 in agreement with the value reported by Okachi *et al.* (1972). The  $K_m$  value for benzylpenicillin was found to be 2.7 mM. The calculated activation energy was 10.9 Kcal/mol corresponding to a temperature coefficient  $Q_{10}$  of 1.85 for the hydrolysis of benzylpenicillin.

The rates of hydrolysis of phenoxymethylpenicillin and ampicillin were 10% and 40%, respectively of the rate with benzylpenicillin (table 2).

**Table 2.** Relative rates of hydrolysis of penicillins by purified *K. citrophila* penicillin acylase.

Substrate	Relative rates of hydrolysis (%)
Benzylpenicillin	100
Phenoxymethylpenicillin	10
Ampicillin	40

Substrates of 0.1 M; 0.1 M potassium phosphate buffer, pH 7.5; temperature 40°C. Relative rates calculated from initial rates of hydrolysis.

The enzyme was stable when stored at  $-20^\circ\text{C}$  and no detectable loss in activity was observed even after a period of 3 months.

### *Immobilization of penicillin acylase*

Data on immobilization of the purified enzyme from *K. citrophila* on CNBr-activated Sepharose 4B and silanized controlled-pore inorganic supports are summarized in table 3.

It is seen from table 3 that protein is quantitatively bound to CNBr-activated Sepharose under the conditions used, the immobilized enzyme showing, however, an efficiency of only 67% on the basis of the activity calculated from the bound protein. This could be due to diffusional restrictions within the gel matrix. Inactivation on chemical coupling is unlikely since penicillin acylase from *E. coli* coupled to CNBr-activated cellulose shows 100% efficiency, the support in this case being non-porous (Subramanian *et al.*, 1978). Under appropriate conditions, a high proportion of the activity (90%) is also picked up from solution by the glutaraldehyde derivative of the silanized controlled-pore  $\text{TiO}_2$ . In the case of enzyme immobilized on  $\text{TiO}_2$ , the

Table 3. Immobilization of *K. citrophila* penicillin acylase.

Supports (initial amount taken)	Soluble enzyme total activity		Immobilized enzyme		
	Amount taken (U)	Amount recovered in filtrate + washings (U)	Activity <sup>a</sup> bound (%)	Specific <sup>b</sup> activity (U/g)	Efficiency (%)
Sephacrose 4B					
(3 g wet wt.)	10	0	100	55 <sup>c</sup>	67
TiO <sub>2</sub> (100 mg)	6.8	3.3	52	32	97
TiO <sub>2</sub> (500 mg)	16.6	1.5	90	28	93
Al <sub>2</sub> O <sub>3</sub> (100 mg)	6.8	3.6	47	25	78
SiO <sub>2</sub> (100 mg)	5.0	0.2	96	30	62.5

Sephacrose was activated with CNBr and the inorganic supports were glutaraldehyde derivatives of the alkylamine carriers.

<sup>a</sup>Calculated from the difference between activity taken and activity recovered in filtrate and washings.

<sup>b</sup>Value obtained on assay.

<sup>c</sup>Calculated value on dry weight basis.

negligible. With Al<sub>2</sub>O<sub>3</sub> and SiO<sub>2</sub> markedly low efficiency values of 78% and 62.5%, respectively, were observed.

#### *Properties of immobilized penicillin acylase*

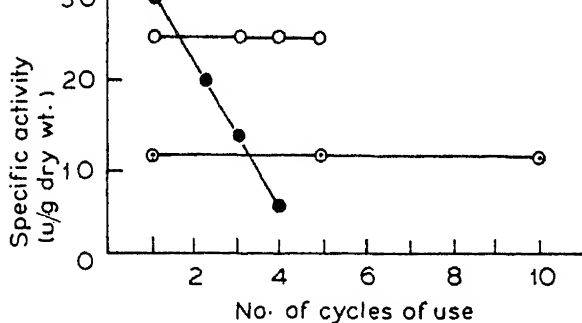
The pH optimum for the hydrolysis of benzylpenicillin by the enzyme immobilized on CNBr-activated Sepharose and on silanized TiO<sub>2</sub> was 7.5, similar to that of the soluble enzyme. The  $K_m$  and activation energy values for the enzyme immobilized on TiO<sub>2</sub> were 2.5 mM and 11.2 Kcal/mol, respectively, which are essentially similar to the values obtained with the soluble enzyme.

#### *Operational stability in the hydrolytic reaction*

The enzyme immobilized on Sepharose was stable over the 22 cycles of use which were carried out, showing no detectable loss in activity in the repeated reuses. Such stability has been reported with *E. coli* penicillin acylase immobilized on CNBr-activated Sephadex G-200 (Ekstrom *et al.*, 1974). Similar results were obtained with the enzyme immobilized on the silanized controlled-pore TiO<sub>2</sub> and Al<sub>2</sub>O<sub>3</sub> over the 10 and 4 cycles, respectively, through which the preparations were used. In marked contrast, the SiO<sub>2</sub> immobilized enzyme lost approximately 20% of its activity in every reuse during the 4 cycles of operation. Figure 2 summarizes the data obtained with the inorganic supports.

#### *Enzymatic synthesis of ampicillin*

Synthesis of ampicillin from 6-APA and DL-phenylglycine methyl ester was

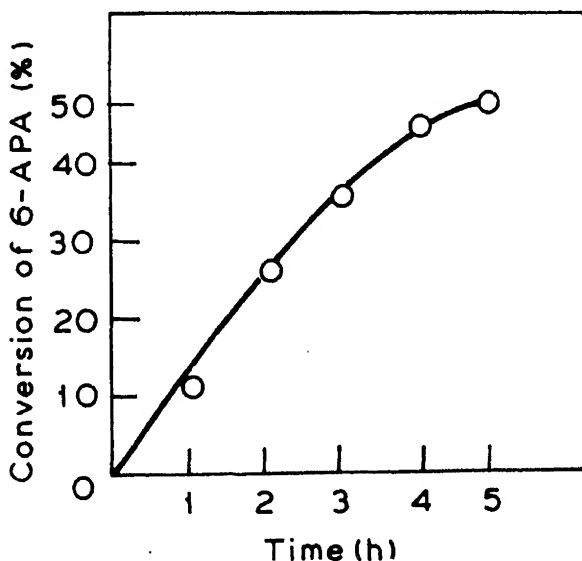


**Figure 2.** Operational stability of *K. citrophila* penicillin acylase immobilized by covalent attachment to silanized controlled-pore ceramics.

(○), Silanized  $\text{TiO}_2$ ; (○), Silanized  $\text{Al}_2\text{O}_3$ ; (●), Silanized  $\text{SiO}_2$ .

immobilized on silanized  $\text{TiO}_2$ . The pH used was 6.5 which has been reported to be the optimum for the synthetic reaction catalysed by *K. citrophila* cells (Okachi *et al.*, 1972). DL-Phenylglycine was taken in 2.5 fold molar excess. The progress curve of ampicillin formation is shown in figure 3.

It can be seen from the curve that approximately 50% conversion of 6-APA is obtained in a period of 5 h under the conditions of the experiment. The reaction mixture at this stage showed the presence of ampicillin and the reactants on reversed



**Figure 3.** Progress curve of formation of ampicillin from 6-APA and DL-phenylglycine methyl ester. Reaction mixture (10 ml) containing 6-APA (50 mg); DL-phenylglycine methyl ester (125 mg); 0.03 M potassium phosphate buffer, pH 6.5, *K. citrophila* penicillin acylase immobilized on silanized  $\text{TiO}_2$  (250 mg support, 7 U). Temperature 40°C.



phase thin layer chromatography. The data presented by Takasawa *et al.* (1972) for whole cells of *K. citrophila* indicate approximately 38% conversion when DL-phenylglycine methyl ester is used in 2.5 fold excess and 66% when D-phenylglycine methyl ester is used.

## Discussion

Controlled-pore ceramics have several desirable features which make them attractive supports for the immobilization of enzymes. Thus they have extreme rigidity, very high surface area, porosity of any desired and narrow range which could exclude microbes and protect the enzyme molecules held within the pores from microbial attack (Messing, 1974). The supports also lend themselves to regeneration after pyrolysis or chemical treatment. Despite the advantages, the use of such supports for the immobilization of penicillin acylase has not been reported hitherto in the literature. The choice of pore size for a particular application depends on the size and shape of the enzyme to be attached, the recommended pore size being approximately twice the large diameter of the enzyme molecule (Messing, 1974). The present studies indicate that controlled pore  $\text{Al}_2\text{O}_3$  of 225 Å pore size has adequate porosity for the binding of *K. citrophila* penicillin acylase of  $M_r \sim 65000$ .

The enzyme immobilized on controlled-pore  $\text{TiO}_2$  has shown 93–97% efficiency compared to the value of 67% obtained with the enzyme bound on Sepharose 4B. The lower value obtained with Sepharose could reflect diffusional restrictions in the case of the gel matrix.

The enzyme immobilized on controlled-pore  $\text{TiO}_2$  and  $\text{Al}_2\text{O}_3$  shows no detectable loss in activity in the limited cycles of use through which the immobilized enzymes were operated. In marked contrast the enzyme bound on controlled pore  $\text{SiO}_2$  loses activity to the extent of about 20% in every cycle of use.

Leakage of glucoamylase covalently attached to silanized  $\text{SiO}_2$  has been reported by Weetall and Havewala (1972), loss of activity being observed even at the acid pH value of 4.5. This was attributed to the dissolution of the glass and the consequent loss of the enzyme bound superficially at the silylated exposed surfaces of the support. The leakage was overcome by them through  $\text{ZrO}_2$ -coating of the support which results in a more resistant surface and stabler  $-\text{Zr}-\text{O}-\text{Silane}$  bonds attaching the enzyme to this surface. The marked loss in activity at pH 7.5 (range pH 7.0–pH 8.0) in every cycle of use of the controlled-pore  $\text{SiO}_2$  immobilized penicillin acylase is probably due to the hydrolytic cleavage of the silyl groups attaching the enzyme to the surface of the support. In contrast the higher stability of the linkage to  $\text{TiO}_2$  and  $\text{Al}_2\text{O}_3$  precludes such a leakage of the attached enzyme.

## Acknowledgement

We are grateful to Howard H. Weetall, Corning Glass Works, USA for gifts of the controlled-pore glass and ceramics.

## References

- Biagi, G. L., Barbaro, A. M., Gapiba, M. F. and Guerra, M. C. (1969) *J. Chromatogr.*, **41**, 371.
- Bomstein, J. and Evans, W. G. (1965) *Anal. Chem.*, **37**, 576.
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.*, **121**, 404.
- Eklstrom, B., Lagerlof, E., Nathorst-Westfelt, L. and Sjoberg, B. (1974) *Sven. Farm. Tidskr.*, **78**, 531.
- Greenstein, J. P. and Winitz, M. (1961) *Chemistry of the Amino Acids*, (New York: John Wiley and Sons) vol. 2, p. 926.
- Kasche, V., Haufler, U. and Zöllner, R. (1984) *Hoppe-Seyler's Z. Physiol. Chem.*, **365**, 1435.
- Lowe, D. A. (1985) *Dev. Ind. Microbiol.*, **26**, 143.
- Lowry, O. H., Rosebrough, N. I., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- March, S. C., Parikh, I. and Cuatrecasas, P. (1974) *Anal. Biochem.*, **69**, 144.
- Messing, R. A. (1974) in *Immobilized Enzymes for Industrial Reactors* (ed. R. A. Messing) (New York: Academic Press) p. 63.
- Okachi, R., Misawa, M., Deguchi, T. and Nara, T. (1972) *Agric. Biol. Chem.*, **36**, 1193.
- Park, J. M., Choi, C. Y., Seong, B. L. and Han, M. H. (1982) *Biotechnol. Bioeng.*, **24**, 1623.
- Ross, G. W. and O'Callaghan, C. H. (1975) *Methods Enzymol.*, **43**, 69.
- Savidge, T. A. (1984) in *Biotechnology of Industrial Antibiotics* (ed. E. J. Vandamme) (New York: Marcel Dekker) p. 171.
- Shimizu, M., Okachi, R., Kumura, K. and Nara, T. (1975) *Agric. Biol. Chem.*, **39**, 1655.
- Subramanian, S. S., Sivaraman, H., Seetarama Rao, B., Ratnaparkhi, R. R. and SivaRaman, C. (1978) *Hind. Antibiot. Bull.*, **20**, 74.
- Takasawa, S., Okachi, R., Kawamoto, I., Yamamoto, M. and Nara, T. (1972) *Agric. Biol. Chem.*, **36**, 1701.
- Vandamme, E. J. and Voets, J. P. (1975) *Experientia*, **31**, 140.
- Weber, K. and Osborn, M. (1969) *J. Biol. Chem.*, **246**, 4406.
- Weetall, H. H. (1976) *Methods Enzymol.*, **44**, 134.
- Weetall, H. H. and Havewala, N. B. (1972) *Biotechnol. Bioeng. Symp.* **3**, Enzyme Engineering (ed. L. B. Wingard) (New York: Interscience Publ.) p. 241.



## Conformational change of L7/L12 stalk in the different functional states of 50S ribosomes

DEBABRATA DASH, SUBODH MAHANTI and DEBI P. BURMA\*

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India

**Abstract.** Conformational change of 50S ribosomes takes place during protein synthesis. The primary change is most likely in the secondary or tertiary structure of rRNA in the L7/L12 stalk region. In order to throw further light on this conformational change, the change in fluorescence of tight couple 50S ribosomes on conversion to loose couple 50S ribosomes containing 5-(iodoacetamido ethyl)-aminonaphthalene-1-sulphonic acid-labelled L7/L12, following the treatment with elongation factor-G and 5'-guanylyl methylene diphosphate was measured. It was enhanced in agreement with the results reported earlier. Further, the quenching of fluorescence of 50S ribosomes containing 5-(iodoacetamido ethyl)-aminonaphthalene-1-sulphonic acid-labelled L7/L12 by acrylamide was studied. The quenching is more in case of loose couples. On conversion of loose couple 50S ribosomes to tight couple ones the quenching becomes less whereas the reverse happens on conversion of tight couple 70S ribosomes to loose couples. These results indicate the conformational change of L7/L12 stalk in the different functional states of 50S ribosomes.

**Keywords.** Ribosomes; protein synthesis; conformation.

### Introduction

It has been demonstrated for the first time in this laboratory that 50S ribosomes undergo conformational change during protein synthesis (Burma *et al.*, 1985a,b; Srivastava and Burma, 1985). Tight couple (TC) 50S ribosomes are converted to loose couple (LC) ones on binding of phetRNA<sup>phc</sup> (Srivastava and Burma, 1985) and subsequently LCs are converted back to TCs during translocation (Burma *et al.*, 1986). It was shown earlier that TC and LC 50S ribosomes differ in the conformations of 23S RNA, most probably in the L7/L12 stalk region (Burma *et al.*, 1984). A new model of translocation was proposed on the basis of this (Burma, 1984; Burma *et al.*, 1985b).

L7/L12 stalk region which is comparatively mobile (Gudkov *et al.*, 1982; Tritton 1978; Van Diggelen *et al.*, 1971), is essential for many functions of ribosomes including translocation (Brot and Weissbach, 1981). In the original model proposed for translocation from this laboratory (Burma, 1984) L7/L12 stalk was arbitrarily put in extended and folded forms in TC and LC 50S ribosomes respectively. Subsequently this was reversed (Burma *et al.*, 1985b) due to the report of Gudkov and Gongadze (1984) that the L7/L12 proteins in 70S elongation factor G (EF-G).5'-guanylyl methylene diphosphate (GMPP(CH<sub>2</sub>)P) complex (ribosomes in the preGTP hydrolysis state) are digested by trypsin whereas in the 70S.EF-G.GDP.fusidic acid

\*To whom all correspondence should be addressed.

Abbreviations used: TC, Tight couple; LC, loose couple; EF-G, elongation factor G; GMPP(CH<sub>2</sub>)P, 5'-

complex (ribosomes in the post GTP hydrolysis state) the L7/L12 proteins are trypsin resistant. This is also in agreement with the crosslinking data of Traut *et al.* (1983). It will be demonstrated in the present communication that the positions of L7/L12 in tight and loose couple 50S ribosomes are directly related to the functional states of 50S ribosomes.

## Materials and methods

### Materials

GTP and fusidic acid were obtained from Sigma Chemical Company, St. Louis, Missouri, USA, polyuridylic acid (Poly(U)) was the product of Miles Laboratory, USA. 5-(iodoacetamido ethyl)-aminonaphthalene-1-sulphonic acid (IAEDANS) was the product of Molecular Probes, USA and obtained as gift from Dr. C. R. Cantor of the Department of Chemistry, Columbia University, New York, USA. Both 5'-guanylyl imidodiphosphate (GMPP(NH)P) and phetRNA<sup>phe</sup> were obtained from Boehringer Mannheim, GMBH, West Germany. Elongation factor-G (EF-G) and elongation factor Tu (EF-Tu) were prepared from S-100 fraction of the extract of *Escherichia coli* MRE 600 according to the method of Gordon *et al.* (1971).

### Methods

*Preparation of TC and LC 50S ribosomes:* TC and LC 50S ribosomes were prepared from 70S ribosomes by ultracentrifugation in the presence of 4 mM Mg<sup>2+</sup> as described earlier (Burma *et al.*, 1985a).

*Preparation of L7/L12 proteins and 70S and 50S core particles:* L7/L12 proteins were extracted from 50S or 70S ribosomes in the presence of 1 M NH<sub>4</sub>Cl and ethanol following the method of Hamel *et al.* (1972) and purified by passing through DEAE-cellulose column according to the method of Moller *et al.* (1972).

*Preparation of IAEDANS-conjugate of L7/L12 proteins:* L7/L12 preparation was dialysed against 50 mM sodium carbonate buffer, pH 9.4 containing 100 mM KCl (buffer A). Dialysed L7/L12 solution (1 mg/ml) was added to a weighed amount of IAEDANS, the molar ratio of dye to protein being 20. After thorough mixing the solution was kept in dark at 30°C for 2–3 h following which it was again extensively dialysed against buffer A. The complete removal of unattached dye was ensured by monitoring the dialysate by fluorescence measurement.

particles were collected by pelleting in high speed swinging bucket rotor of Beckman L5 Ultracentrifuge. The pelleting process was repeated to remove the unattached dye conjugate of proteins.

*Fluorescence measurement:* The fluorescence was measured in a total volume of 0.3 ml containing 10 mM Tris-HCl, pH 7.5, 25 mM magnesium acetate, 100 mM KCl, 6 mM  $\beta$ -mercaptoethanol and 1  $A_{260}$  unit of 50S ribosomes or 1.5  $A_{260}$  units of 70S ribosomes as described by Lee *et al.* (1981a,b) in a Perkin-Elmer LS-5 Luminescence spectrophotometer using quartz micro cell of 5 mm path length. The excitation was done at 360 nm and the emission spectrum was recorded in each case. When acrylamide (recrystallised from chloroform before use) was used as a quencher requisite amounts of 2 M acrylamide solution were added step by step to the contents of the cell (0.3 ml).

*Conversion of LC to TC 50S ribosomes containing IAEDANS-labelled L7/L12:* The procedure was the same as described by Burma *et al.* (1985a) for unlabelled ribosomes. LC 50S ribosomes (40 pmol) were treated at 37°C for 30 min with EF-G (200 pmol), GTP (2.5 nmol) and fusidic acid (5 mM) in a total volume of 0.2 ml of buffer C containing 50 mM Tris-HCl, pH 7.5, 160 mM  $NH_4Cl$ , 10 mM  $Mg^{2+}$  and 12 nM  $\beta$ -mercaptoethanol. Subsequently the mixture was cooled to 4°C and diluted to 0.3 ml with buffer C.

*Conversion of TC to LC 50S ribosomes containing IAEDANS-labelled L7/L12:* The conversion of TC 50S ribosomes to LC 50S ribosomes was carried out at the level of 70S ribosomes (Burma *et al.*, 1985a, 1986). In the first case the incubation was carried out at 37°C for 30 min in 0.2 ml of 50 mM Tris-HCl, pH 7.5 containing 160 mM  $NH_4Cl$ , 10 mM  $Mg^{2+}$  and 1 mM dithiothreitol having TC 50S ribosomes (40 pmol), equivalent amount of 30S ribosomes, EF-G (200 pmol), poly(U) (8  $\mu g$ ) and GMPP(NH)P (0.8 nmol). After cooling to 4°C the incubation mixture was diluted to 0.8 ml with the above-mentioned buffer. In the second case TC 50S ribosomes (40 pmol) along with equimolar amount of 30S ribosomes were treated at 25°C for 20 min with 8  $\mu g$  of poly(U), 80 pmol of EF-Tu, 0.25 mM GTP and 80 pmol of phetRNA<sup>phe</sup> in a total volume of 0.2 ml containing 50 mM Tris-HCl, pH 7.6, 80 mM KCl, 80 mM  $NH_4Cl$ , 5 mM dithiothreitol and 8 mM  $Mg^{2+}$ . After incubation this mixture was cooled to 4°C and diluted to 0.3 ml with the same buffer.

## Results

### *Effect of interconversion of tight and loose couple 50S ribosomes on the fluorescence of 50S ribosomes containing IAEDANS-labelled L7/L12*

It has already been demonstrated in this laboratory that the fluorescence of LC 50S ribosomes containing IAEDANS-labelled L7/L12 is enhanced on treatment with EF-G, GTP and fusidic acid (Burma *et al.*, 1986). However, on treatment of TC

be reduced. The former treatment leads to the conversion of TC to LC 50S ribosomes while the reverse happens in the latter case. These results can be rationalised if it is assumed that the L7/L12 stalk is in an extended form in LC 50S ribosomes and more towards the body of ribosomes in the tight couples. It has been shown earlier that TC ribosomes can be converted to LC ribosomes on treatment with EF-G and a GTP analogue, GMPP(CH<sub>2</sub>)P (Burma *et al.*, 1985a). Therefore IAEDANS-labelled L7/L12 were incorporated into TC 50S ribosomes essentially following the method of Lee *et al.* (1981a,b). On treatment of TC 70S ribosomes containing IAEDANS-labelled L7/L12, with EF-G and GMPP(NH)P the fluorescence becomes reduced (figure 1). It should be noted that the reduced value is somewhat more than that of LC 50S ribosomes. This is expected as the conversion is known to be approximately 50–60% (Burma *et al.*, 1985a). These results also support the earlier assumption that the L7/L12 stalk is in an extended form in LC 50S ribosomes and folded form in TC 50S ribosomes. This was found to be true by using acrylamide as a quencher as described below.

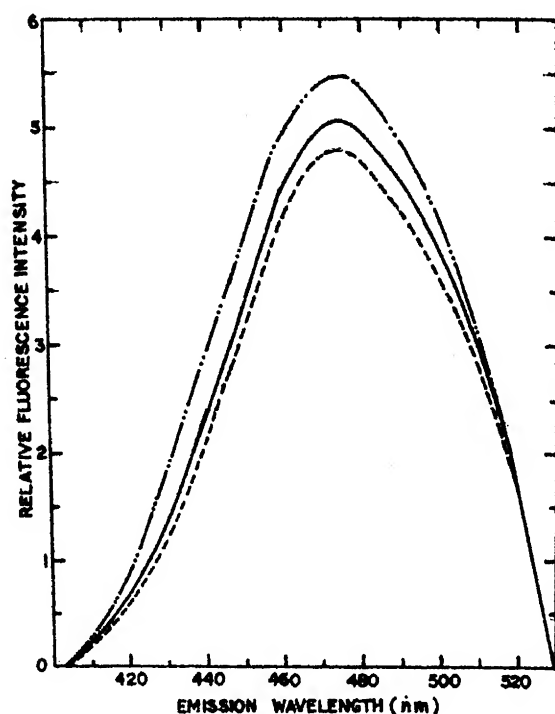


Figure 1. Quenching of fluorescence of 50S ribosomes containing IAEDANS-labelled L7/L12, on treatment with EF-G and GMPP(NH)P. The preparation of 50S ribosomes having IAEDANS-labelled L7/L12 has been described under 'materials and methods'. The method of measurement of fluorescence has also been described under 'materials and methods'.

(—) TC 70S ribosomes; (---) LC 50S ribosomes; (- · -) TC 70S ribosomes treated with

The quenching of fluorescence of TC and LC 50S and 70S ribosomes containing IAEDANS-labelled L7/L12 has been recorded in figure 2 as ratio of fluorescence at 475 nm in the presence and absence of acrylamide against the concentration of the quencher. The slopes of the curves indicate  $K_q\tau_o$  values where  $K_q$  represents the association constant of the quencher with IAEDANS and  $\tau_o$  is the lifetime of fluorophor (in ns). The values turn out to be 7.27 (50S LC), 6.52 (50S TC), 6.85 (70S LC) and 6.00 (70S TC). These were fairly constant in a number of measurements. Unfortunately, it was not possible for us to measure  $\tau_o$  value but  $\tau_o$  is practically constant as it appears from the values reported by Lee *et al.* (1981b). If it is so, it may be concluded that IAEDANS is more available for collision with acrylamide in 50S LC than TC ribosomes. As expected, the same is true in case of 50S ribosomes in comparison to 70S ribosomes. Therefore these data indicate that L7/L12 stalk is more exposed in LC than TC ribosomes.

*Interconversion of tight and loose couple ribosomes and change of fluorescence of 50S ribosomes containing IAEDANS-labelled L7/L12*

As mentioned already, LC 50S ribosomes are converted to TC 50S ribosomes on treatment with EF-G, GTP and fusidic acid whereas TC 50S ribosomes are converted to LC 50S ribosomes on treatment with EF-G and a GTP analogue (Burma *et al.*, 1985a). Further, similar conversion of TCs to LCs can be effected on treatment with phetRNA<sup>phe</sup>, EF-Tu and GTP in the presence of poly(U). The effects of such conversion on the quenching by acrylamide of the fluorescence of TC and LC 50S ribosomes containing IAEDANS-labelled L7/L12 are shown in table 1.  $K_q\tau_o$  values are the slopes of the linear plot of  $F_o/F$  against  $[Q]$ , as discussed above and averages of 3 independent measurements (figure 2). When LC 50S ribosomes are converted to TC 50S ribosomes with EF-G, GTP and fusidic acid  $K_q\tau_o$  value approaches from that of LC to TC (table 1). It is already known that the conversion is 90–95% under such condition (Burma *et al.*, 1985a). When TC 70S ribosomes were converted to LC 70S ribosomes in the presence of EF-G and GMPP(NH)P the value after conversion of TC to LC 50S ribosomes is much less. When TC 70S ribosomes are treated with phetRNA<sup>phe</sup> in the presence of poly(U), EF-Tu and GTP the fluorescence is 70% of the final products (table 1). Thus the quenching of fluorescence corroborates the findings recorded earlier (Burma *et al.*, 1985a, 1986; Srivastava and Burma 1985).

## Discussion

Ribosomes are known to exist in two different forms, tight and loose couples. TC ribosomal subunits (30S and 50S) do not dissociate at low  $Mg^{2+}$  concentration (4 mM or so) whereas LCs readily dissociate at this  $Mg^{2+}$  concentration. LC ribosomes are much less biologically active and thought to be damaged ones (Noll *et al.*, 1973a, b; Hapke and Noll, 1976). It was shown for the first time in this laboratory that loose couples can be readily converted to biologically active tight couples on



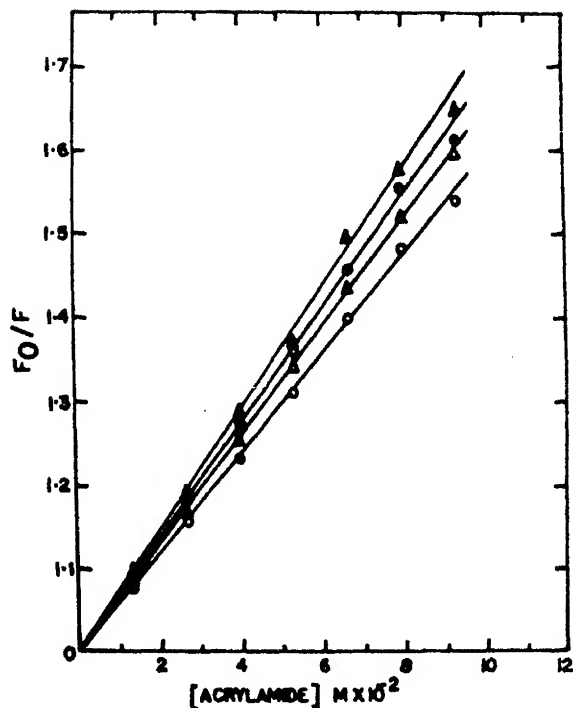


Figure 2. Quenching of fluorescence of IAEDANS-L7/L12-ribosomes by acrylamide. The method of measurement of fluorescence of 50S and 70S ribosomes having IAEDANS-labelled L7/L12 in presence ( $F$ ) and absence ( $F_0$ ) of acrylamide used as quencher has been described under 'materials and methods'. Straight lines have been drawn by the least square method.

(▲) LC 50S Ribosomes; (●) LC 70S ribosomes; (Δ) TC 50S ribosomes; (○) TC 70S ribosomes.

(Burma *et al.*, 1985a). Similarly TCs can be converted to LCs on treatment with EF-G and a GTP analogue (GMPP(NH)P or GMPP(CH<sub>2</sub>)P). It has been further shown in this laboratory that TCs are converted to LCs on treatment with phetRNA<sup>phe</sup>, EF-Tu and GTP (Srivastava and Burma, 1985). On the basis of the above-mentioned data it has been proposed (Burma, 1984; Burma *et al.*, 1985b) that LCs are not damaged ribosomes but actually 'intermediate' in protein synthesis. It has been further suggested that protein synthesis is initiated with TCs and during the binding of aminoacyl tRNA they are converted to LCs. However, during translocation of aminoacyl tRNA along with mRNA, LCs are converted back to TCs (Burma *et al.*, 1986). This cyclic process has been shown in figure 3.

It was first shown by Van Diggelen *et al.* (1971) that the differences between TC and LC ribosomes lie in the 50S subunits. It was unequivocally demonstrated in this laboratory that 50S ribosomes exist in two different conformations and that also due to two different conformations of 23S RNA (Burma *et al.*, 1984). Various studies also indicate that the conformational differences between TC and LC 50S subunits are RNA

**Table 1.** Interconversion of tight and loose couple ribosomes as indicated by the quenching of fluorescence of IAEDANS-L7/L12-ribosomes.

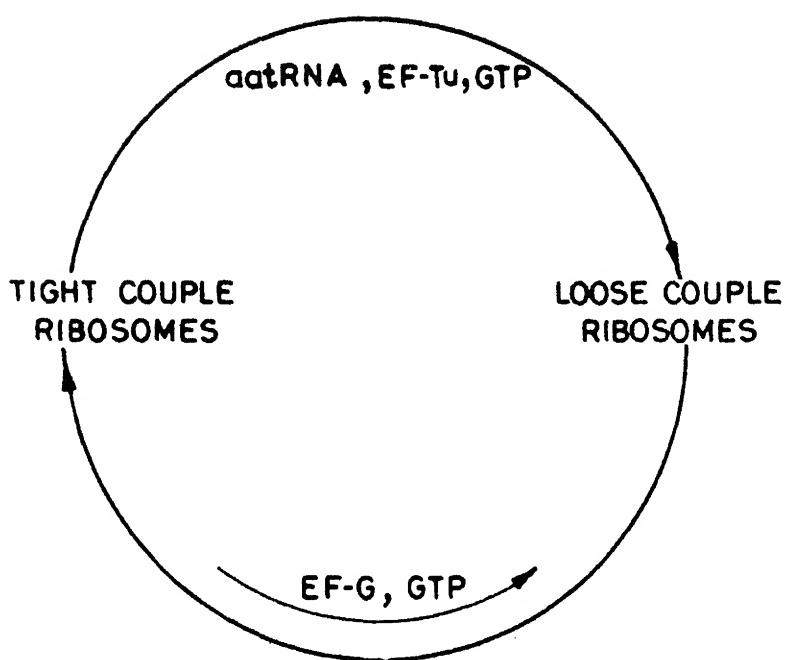
Ribosomal preparations	$K_q \tau_o$
LC 50S ribosomes	7.33
LC 50S ribosomes (treated with EF-G, GTP and fusidic acid)	6.66
TC 50S ribosomes	6.46
TC 70S ribosomes	6.00
TC 70S ribosomes (treated with EF-G and GMPP(NH)P)	6.44
LC 70S ribosomes	6.95
TC 70S ribosomes	6.00
TC 70S ribosomes (treated with EF-Tu and phetRNA <sup>phe</sup> in presence of poly(U))	6.61
LC 70S ribosomes	6.94

The methods of treatment have been described under 'materials and methods'. The ratio of arbitrary fluorescence values in the presence and absence of acrylamide used as quencher was plotted against the concentration of the quencher as in figure 2.  $K_q \tau_o$  values are the slopes of linear plots. The details have been described under 'materials and methods'.

conformation in this region, the switch over being controlled by EF-G and GTP (Burma, 1984; Burma *et al.*, 1985b, 1986).

L7/L12 proteins which occur in 4 copies, constituting the stalk region of 50S ribosomes, are known to be involved not only in translocation but also many other steps of protein synthesis (for review see Brot and Weissbach, 1981). These proteins are known to be mobile (Kischa *et al.*, 1971; Tritton, 1978; Gudkov *et al.*, 1982). In the model proposed from this laboratory for translocation in protein synthesis it has been assumed that L7/L12 proteins are responsible for the folding and unfolding of rRNA in this region (Burma, 1984; Burma *et al.*, 1985b, 1986). The assumption is strongly supported by the data presented in this paper.

Lee *et al.* (1981a, b) first demonstrated that there is quenching of fluorescence of L7-IAEDANS-70S ribosomes in the presence of poly(U) and phetRNA<sup>phe</sup>. This indicated the change in conformation of 50S ribosomes, specially in the L7/L12 stalk region. Similar fluorescence studies in this laboratory indicated the position of L7/L12 in more hydrophobic region in the TC 50S ribosomes than LC 50S ribosomes (Burma *et al.*, 1986). The present studies using acrylamide as quencher also support the above contention. These data indicate further that the L7/L12 stalk region is close to the main body of 50S ribosomes (representing TC 50S ribosomes) in the initial stage of protein synthesis. However, the alternate explanation of differential quenching due to differences in relative positions of L7/L12 dimers in the stalk region of 50S ribosomes cannot be ruled out. Work is in progress to understand the positions and involvement of L7/L12 during translocation. Anisotropic measure-



**Figure 3.** Cyclic process of conversion of tight and loose couple 50S ribosomes during protein synthesis.

## Acknowledgements

The financial assistance was received from the University Grants Commission, Council of Scientific and Industrial Research and the Department of Science and Technology, New Delhi. IAEDANS was a gift from Dr Charles Cantor, Columbia University, New York, USA. Thanks are also due to him for some valuable suggestions.

## References

- Brot, N. and Weissbach, H. (1981) *Mol. Cell. Biochem.*, **36**, 47.  
 Burma, D. P. (1984) *J. Biosci.*, **6**, 419.  
 Burma, D. P., Srivastava, A. K., Srivastava, S., Tewari, D. S., Dash, D. and Sengupta, S. K. (1984) *Biochem. Biophys. Res. Commun.*, **124**, 970.  
 Burma, D. P., Srivastava, A. K., Srivastava, S. and Dash, D. (1985a) *J. Biol. Chem.*, **260**, 10517.  
 Burma, D. P., Srivastava, A. K., Srivastava, S., Dash, D., Tewari, D. S. and Nag, B. (1985b) *Proc. Int. Symp. Biomol. Struct. Interactions Suppl. J. Biosci.*, **8**, 767.  
 Burma, D. P., Srivastava, S., Srivastava, A. K., Mahanti, S. and Dash, D. (1986) in *Ribosomes, structure, function and genetics* (ed. B. Hardesty) (New York: Springer Verlag) p. 438.  
 Gordon, J., Lucas-Lenard, J. and Lipmann, F. (1971) *Methods Enzymol.*, **20**, 281.  
 Gudkov, A. T. and Gongadze, G. M. (1984) *FEBS Lett.*, **176**, 32.  
 Gudkov, A. T., Gongadze, G. M., Bushuev, B. N. and Okon, M. S. (1982) *FEBS Lett.*, **138**, 229.  
 Hamel, E., Koka, M. and Nakamoto, T. (1972) *J. Biol. Chem.*, **247**, 805.  
 Hapke, B. and Noll, H. (1976) *J. Mol. Biol.*, **105**, 97.

- Lee, C. C., Wells, B. D., Fairclough, R. H. and Cantor, C. R., (1981b) *J. Biol. Chem.*, **256**, 49.
- Moller, W., Groene, A., Terhorst, C. and Amons, R., (1972) *Eur. J. Biochem.*, **25**, 805.
- Noll, H., Noll, M., Hapke, B. and Van Diejien, G., (1973a) in *Regulation of transcription and translation in Eukaryotes* (ed. E. Bautz) p. 257.
- Noll, M., Hapke, B. and Noll, H. (1973b) *J. Mol. Biol.*, **80**, 519.
- Srivastava, S. and Burma, D. P. (1985) *FEBS Lett.*, **186**, 17.
- Traut, R. R., Lambert, J. M. and Kenny, J. W. (1983) *J. Biol. Chem.*, **258**, 14592.
- Tritton, T. R. (1978) *Biochemistry*, **17**, 3959.
- Van Diggelen, O. P., Heinsius, H. L., Kalousek, F. and Bosch, L. (1971) *J. Mol. Biol.*, **55**, 277.



# Arginine decarboxylase is a component activity of the multifunctional enzyme putrescine synthase in cucumber seedlings

G. L. PRASAD and P. R. ADIGA\*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

**Abstract.** A homogenous preparation of putrescine synthase, the versatile multifunctional enzyme involved in agmatine→putrescine conversion in *Cucumis sativus* was found to catalyze enzymatic decarboxylation of arginine also. Similarly, the purified arginine decarboxylase mediated the component as well as the complete set of coupled reactions harboured by putrescine synthase. Both the enzyme preparations exhibited identical electrophoretic and chromatographic behaviour and were immunologically indistinguishable. All the enzymic activities are stabilized concurrently by feeding arginine to the intact seedlings. Therefore, it is concluded that the multifunctional putrescine synthase in *Cucumis sativus* seedlings also harbours arginine decarboxylase activity unlike its counterpart in *Lathyrus sativus*.

**Keywords.** Putrescine biosynthesis; arginine decarboxylase; multifunctional enzyme.

## Introduction

Earlier studies in this laboratory (Adiga and Prasad, 1985) on enzymatic aspects of polyamine biosynthesis in higher plant systems have led to purification and characterisation of the first enzyme of the biosynthetic pathway *viz.*, arginine decarboxylase (ADC, EC 4.1.1.19), the multifunctional enzyme putrescine synthase (PS) involved in agmatine→putrescine conversion from the seedlings of *Lathyrus sativus* (Ramakrishna and Adiga, 1975; Srivenugopal and Adiga, 1981) as well as *Cucumis sativus* (Prasad and Adiga, 1985, 1986b). The molecular characteristics of these two enzymes from the above plant sources were found to differ significantly in terms of finer details of their structural and functional characteristics. Thus, while ADC from *L. sativus* did not catalyze ornithine decarboxylation, the cucumber enzyme exhibited both these activities (Prasad and Adiga, 1986a). Furthermore, despite the fact that the polycephalic enzyme, PS from two plant species differed structurally, the cucumber enzyme nevertheless harboured as expected all the 4 constituent activities *viz.*, agmatine iminohydrolase (AIHase, EC 3.5.3.12), ornithine transcarbamylase (OTCase, EC 2.1.3.3), putrescine transcarbamylase (PTCase, EC 2.1.3.6) and carbamate kinase (CKase, EC 2.7.2.2) and the complete reaction resulting in agmatine→putrescine transformation as the direct consequence of coupling either OTCase or CKase activities to PTCase component of PS. During these studies it was observed rather unexpectedly, that the purified ADC and PS of cucumber seedlings exhibited identical chromatographic and electrophoretic properties. Hence a detailed investigation was undertaken to probe in to the discrete possibility that each of the two enzyme preparations purified through two distinct

\*To whom all correspondence should be addressed.

Abbreviations used: ADC, Arginine decarboxylase; PS, putrescine synthase; AIHase, agmatine

protocols, catalyze the reactions catalyzed by the other. This paper deals with these investigations which support the concept that in *C. sativus* seedlings, ADC activity is also integral constituent of the multifunctional putrescine synthase.

## Materials and methods

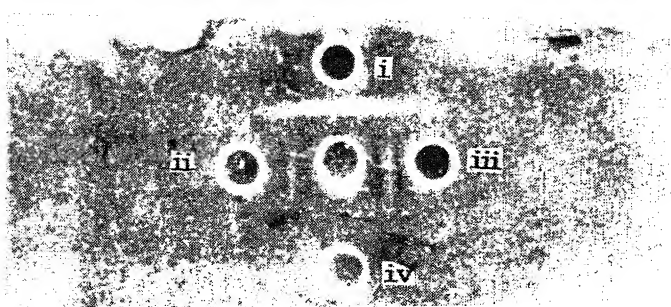
The source of the seeds and growth conditions of *C. sativus* seedlings and the reagents used have been described in detail earlier (Prasad and Adiga, 1985). The enzyme ADC and PS have been purified from *C. sativus* seedlings as detailed elsewhere (Prasad and Adiga, 1985b, 1986b). The ADC was purified by a 3-step procedure involving ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-150. PS was isolated by affinity chromatography on putrescine carboxyhexyl Sepharose. ADC activity was quantified by measuring the CO<sub>2</sub> evolved when [U-<sup>14</sup>C]-arginine was used as the substrate in Warburg flasks (Prasad and Adiga, 1985b). The constituent and the complete reactions catalyzed by PS were assayed according to Prasad and Adiga (1986b). Ouchterlony immunodouble diffusion (Ouchterlony, 1967), polyacrylamide gel electrophoresis (PAGE) on non-denaturing slab gels (Davis, 1964) and on denaturing gels (Laemmli, 1970) were performed according to the standard procedures. Protein estimation using bovine serum albumin as the standard was carried out according to Lowry *et al.* (1951) and the amines were estimated in the deproteinized enzyme reaction mixtures by the standardized procedures employed earlier (Srivenugopal and Adiga, 1981; Prasad and Adiga, 1986b).

### Unit activity

Unless otherwise stated, one unit of enzyme activity is defined as the amount of enzyme required to produce 1  $\mu$ mol of product (NCP citrulline or ATP) under standard assay conditions. Specific activity is expressed in units/mg protein.

## Results

A careful scrutiny of the behaviour of ADC and PS of cucumber during purification revealed that both of them behave identically during ion-exchange chromatography, gel filtration and during electrophoresis on non-denaturing as well as denaturing polyacrylamide gels (Prasad and Adiga, 1985b, 1986b). Thus both, these enzyme activities are elutable with about 0.2 M KCl at pH 7.6 from DEAE-cellulose columns and both are excluded in the void volume of Sephacryl S-300 gel filtration columns. Furthermore, the two enzymes comigrate as single species on non-denaturing polyacrylamide gels at pH 8.3, yet resolve into 48 K, 44 K and 15 K polypeptides under reducing conditions on sodium dodecyl sulphate (SDS)-PAGE. Under non-reducing conditions these two enzyme preparations are found to band with an apparent molecular mass of 150 KD during SDS-PAGE (data not given). In line with these similarities, homogenous ADC preparation could cross-react with the immunoglobulin fraction prepared from the anti-serum raised against PS showing thereby that they share immunological cross-reactivity (figure 1). All these observations have



**Figure 1.** Cross-reactivity of ADC with specific immunoglobulin-G against putrescine synthase.

Wells (i) and (ii) ADC, (iii) PS, (iv) BSA and centre well contained immunoglobulin-G.

### *Product isolation studies*

A more convincing proof for the above premise stems from the product isolation from enzyme reaction mixtures. When the amine products from ADC reaction mixture were isolated and separated on paper chromatograms, it could be shown that putrescine, rather than agmatine accounts for more than 90% of the product derived from arginine decarboxylation (table 1). This unexpected, yet intriguing finding could be further confirmed by using non-radioactive arginine as the substrate at saturating concentrations and identifying ninhydrin-positive putrescine on the chromatograms (data not shown). Addition of inorganic phosphate, which was earlier shown to enhance ADC activity (Prasad and Adiga, 1986a), gave rise to enhanced production of putrescine. Inclusion of agmatine (10 mM), the immediate product of arginine decarboxylation, in the ADC assay mixture could trap significant amount of radioactivity in agmatine fraction, subsequently purified on paper chromatograms; nevertheless significant amount of radioactive putrescine could still be recovered as one of the products under these conditions.

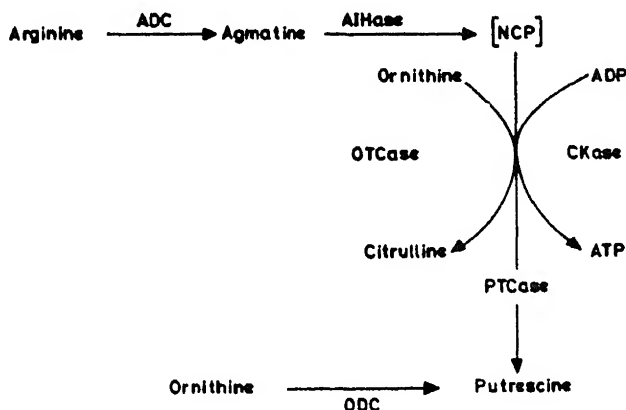
**Table 1.** Identification of product amines in ADC reaction mixture. Enzyme source: Pure ADC (Sephadex G-150 eluate).

Additions (mM)	Radioactivity (cpm)	
	Putrescine	Agmatine
Nil	2,050	250
(Complete system)		
$P_i$ (5)	5,300	500
$P_i$ (5)+ agmatine(10)	3,300	1,300

The enzyme reaction was carried out according to Prasad and Adiga (1985b).  $P_i$  or  $P_i$  + agmatine were included in the standard assay mixture at indicated concentrations. ADC activity was



The enzymatic conversion of NCP→putrescine as catalyzed by PTCase has been earlier shown to be thermodynamically unfavourable (Srivenugopal and Adiga, 1981) (for enzymatic reactions, see figure 2). It may be recalled that in order to drive the reaction in favour of putrescine formation, the putrescine synthase utilizes two coupled enzymatic reactions, involving either OTCase or CKase. However, the results of the present study show that under the assay conditions employed, the arginine→putrescine conversion, which occurs *via* agmatine and NCP, can proceed even in the absence of either added ornithine or ADP +  $P_i$ . This observation is in apparent contradiction with that made with *L. sativus* PS (Srivenugopal and Adiga, 1981). Of relevance in this context, are the earlier reports that decarboxylation reaction could yield energy of  $\approx 7$  K cal (Jencks, 1970). Therefore, an attempt was made to investigate whether the decarboxylation of arginine is also an exergonic reaction, in which case the thermodynamic constraint referred to above could be overcome and the putrescine production from arginine by the multifunctional enzyme be easily explained.



**Figure 2.** Reactions catalyzed by PS from cucumber seedlings.

Putrescine synthase from *C. sativus* in addition to mediating agmatine→putrescine conversion, unlike its counterpart from *L. sativus* (Srivenugopal and Adiga, 1981), also catalyzes decarboxylation of arginine and ornithine.

Data of table 2, clearly indicate that the decarboxylation can be coupled to the production of ATP and that at least a major part of the energy generated during arginine decarboxylation could be conserved. Production of ATP was maximum when ornithine and agmatine were included in the assay mixture. However, addition of ornithine alone decreased ATP generation, presumably by competing with arginine as observed earlier (Prasad and Adiga, 1985b). It may be noted that agmatine which is inhibitory to ADC activity, also decreased the yield of ATP. It could be shown that the  $K^+$  and  $Mg^{2+}$  ions are required for optimal activity and are probably involved at the level of coupling of the component activities or kination, i.e. at PTCase. This inhibition is not observed with other field host metabolites (Prasad

**Table 2.** Decarboxylation of arginine coupled to ATP synthesis (catalyzed by pure ADC preparation).

Additions (mM)	Activity units (nmol of ATP produced/mg protein)
Arginine(5)	157.5
Arginine(5) +	
Agmatine(5)	65.6
Arginine(5) +	
Ornithine(5)	0
Arginine(5) - $Mg^{2+}$	109.0
Arginine(5) - KCl	97.5
Agmatine(5) +	
Ornithine(5)	341.2
Agmatine(5) +	
Ornithine(5) - $Mg^{2+}$	223.0

The ADC assay was carried out as described in table 1. Indicated additions were made to the ADC reaction mixture prior to the commencement of assay. Standard assay mixture also consisted of 2 mM ADP; 5 mM  $P_i$ , 5 mM  $Mg^{2+}$  and 1 mM  $K^+$ . After terminating the ADC reaction, the ATP generated was assayed by hexokinase-glucose-6-phosphate dehydrogenase coupled assay as described by Srivenugopal and Adiga (1981).

#### *Agmatine → putrescine conversion by ADC preparation*

The complete reaction which is a measure of the activities catalyzed by PS (agmatine + ornithine  $\xrightarrow{P_i}$  putrescine + citrulline), was also mediated by the ADC preparation. This reaction also displayed the same requirements as those by the reaction mediated by PS (table 3).

#### *Decarboxylation of arginine catalyzed by PS*

Since all the above reactions were observed with ADC preparation, it was considered necessary to demonstrate decarboxylation of arginine by purified PS also. For this purpose, the putrescine-Sepharose eluate was employed. As expected, it could be clearly shown that putrescine-Sepharose eluates decarboxylated arginine enzymatically and efficiently and this was inhibited by agmatine (table 4), a phenomenon demonstrated earlier with purified ADC.

linked to OTCase activity catalyzed by pure ADC preparation (Sephadex G-150 eluate).

Agmatine + ornithine  $\xrightarrow{P_i}$  citrulline + putrescine.

Assay condition	Activity units (nmol of citrulline produced)/mg protein
Complete system*	206
- $P_i$	6
+ $P_i$ (2 mM)	100
- Ornithine	5
- Agmatine	0

\*Complete system contains: Tris-HCl pH 8.8; 50  $\mu$ mol; agmatine 2.5 mM; ornithine 0.5 mM;  $Mg^{2+}$  10 mM;  $P_i$  5 mM; enzyme 80  $\mu$ g.

Purified ADC was used as the enzyme source. Citrulline was quantitated (Prasad and Adiga, 1986b).

**Table 4.** Decarboxylation of arginine by PS (putrescine-CH-Sepharose eluate).

Enzyme protein added ( $\mu$ g)	Additions	Activity units*
12	Nil	2.4
48	Nil	8.3
48	Agmatine	2.2(75)

The value in parenthesis is percent inhibition.  
\*picomoles of  $^{14}CO_2$  liberated in a 2 h assay.  
Agmatine (10 mM) was included to the standard assay mixture prior to ADC assay.

All the component activities tested were stabilized upon feeding with arginine. Day 3 seedlings which were exposed to arginine for a longer time exhibited maximum activities, followed by day 5 seedlings. Untreated seedlings processed on day 8 had relatively the least activity (table 5).

### Product switch

Analysis of the total amine fraction of cucumber seedlings showed the presence of significant amounts of agmatine along with spermidine, spermine and putrescine (Suresh *et al.*, 1978). If the cucumber PS converts arginine to putrescine, through a series of coupled reactions then the accumulation of agmatine in significant amounts in the seedlings cannot be easily explained. Towards this end, yet another regulatory feature of the cucumber enzyme became evident, while assessing the stoichiometry of the ADC reaction. With crude enzyme extracts, the major product of the arginine

from cucumber seedlings.

Enzyme activity	Activity of control (%)	
	Arginine fed for 3 days	Arginine fed for 5 days
ADC*		
(arginine→agmatine + CO <sub>2</sub> )	130	253
Complete reaction (agmatine + ornithine→citrulline + putrescine)	111	285
OTCase (ornithine + carbamyl-phosphate →citrulline)	117	182
PTCase (putrescine + carbamyl-phosphate →NCP)	150	200

Untreated, 8-day-old seedlings served as control. Enzyme activities were assayed in crude extracts.

\*From the data presented it is evident that ADC is also a component activity of PS in *C. sativus*.

**Table 6.** Identification of product amines in ADC reaction mixture. Enzyme source: Crude extracts.

Enzyme source	Radioactivity (cpm)	
	Putrescine	Agmatine
Control	1980	8250
Homoarginine	5610	21450
KCl	990	1485

Cotyledons were treated either with homoarginine (10 mM) for 12 h, or K<sup>+</sup> (30 mM for 72 h) and crude ADC was prepared as described by Prasad and Adiga (1985a). ADC assay was carried out as described by Prasad and Adiga (1985a). Product amines were isolated from the reaction mixture as already detailed.

with which putrescine is the major end product. This was found to be the case when the cotyledons were cultured with homoarginine and KCl also (table 6). It is clear that the switch over from agmatine→putrescine as the major product of the reaction occurs as a result of purification of the enzyme with the elimination of other controlling factors which presumably modulate the *in vivo* elaboration of the various amine intermediates.

## Discussion

The multifunctional enzyme, PS of cucumber seedlings exhibits higher degree of structural complexity than its *L. sativus* counterpart (Prasad and Adiga, 1986b).

also. Thus it appears that in cucumber, the whole of biosynthetic machinery required for the biogenesis of putrescine is organized into a single yet multifunctional unit in the form of PS. This rather unexpected finding has been supported by several pieces of circumstantial evidence such as; (i) identical elution pattern of ADC and putrescine-Sephadex eluate activities during purification by ion-exchange and gel filtration chromatography, (ii) indistinguishable electrophoretic behaviour on non-denaturing and denaturing gels, (iii) complete immunological cross-reactivity, (iv) susceptibility to proteolysis and (v) co-ordinate stabilization of the activities in the seedlings by feeding arginine. Unequivocal proof favouring ADC as the constituent activity of putrescine as the sole product of enzymic reaction catalysed by the purified ADC which sharply contrasts with the situation observed with the *L. sativus* system.

A major question that stems from such a situation is concerned with the nature and source of the driving force required to 'push' the energetically unfavourable phosphorylitic cleavage of NCP→putrescine in the absence of added other substrates to support the coupling reactions. The demonstration that enzymatic decarboxylation of arginine *per se* could meet this energy demand is evident from the data of table 2. This accords with the postulate of Rabinovitch and Flowers (1964) with regard to the functioning of multifunctional/multienzyme systems in that the energy generated in a reaction could be utilized by the subsequent one. It is noteworthy that when either agmatine or NCP acts as the substrate, the PTCase should obligatorily be coupled to either OTCase or CKase ensure substantial putrescine production.

Another important issue that merits attention is the *in vivo* accumulation of agmatine in the plant, which is in apparent contradiction to the data discussed above. However, the enzyme in the crude extracts, gives rise to agmatine from arginine, while in purified form produces putrescine (tables 6 and 1). It would, therefore appear, that the AIHase activity of the multifunctional enzyme remains partly suppressed in the crude extracts, presumably by a regulator, that determines the extent of conversion of agmatine→putrescine, through NCP, while accounting for the release of agmatine into the reaction medium. Our unpublished results (Prasad and Adiga) indicate that the regulator is lost at the DEAE-cellulose chromatography step during purification. An analogous situation is the finding that in *Salmonella typhimurium*, prephenate produced by the action of chorismate mutase component of chorismate mutase-prephenate dehydratase complex has been shown to diffuse into the medium before entering prephenate dehydratase site for further metabolism (Schmit and Zalkin, 1969). If such a regulator for AIHase exists and is functional *in vivo*, then AIHase reaction assume the role of the 'committed step' in putrescine biosynthesis. In other words, although ADC happens to be the rate limiting step, agmatine produced may not be obligatorily converted *in toto* to putrescine, not withstanding the fact that the whole of the biosynthetic machinery for the diamine is organized into a single enzyme. Vance (1976) has argued that multifunctional enzymes have distinct advantages in catalyzing those metabolic sequences wherein the rate limiting reaction is not the committed step, so that the unnecessary accumulation or intermediates is prevented. This kind of situation is reminiscent of the 'Sluice gate' type regulation discussed for the arom conjugate of *Neurospora*

*crassa* (Welch, 1977; Welch and Gaertner, 1980). In terms of cellular economy, the benefits that accrue to the plant by recruiting the multifunctional PS for arginine→putrescine conversion apparently arise from channelling the intermediate metabolites through the coupled reactions discussed earlier (Srivenugopal and Adiga, 1981).

From the foregoing, it is abundantly clear that the putrescine synthase of *C. sativus* significantly differs from its *L. sativus* counterpart, despite the fact that both the enzymes mediate the diamine biosynthesis. It is pertinent to mention in this context, that the enzyme 'arom' conjugate investigated in detail in *N. crassa* also exhibits a broad spectrum of differential association of constituent activities in several other organisms hitherto examined (Welch and Gaertner, 1980). Similarly, the anthranilate synthase is bifunctional in some microbes, whereas in several others it is monofunctional (Zalkin, 1980). Another classical example worthy of attention in this context is the different enzyme activities associated with the fatty acid synthase system (Schewizer, 1980; Wakil *et al.*, 1983). Whatever may be its ultimate organisational complexity, it is clear from the foregoing that the PS from the cucumber seedlings is a highly versatile multifunctional protein and a comprehensive appreciation of its catalytic, structural and regulatory aspects needs a clearer understanding of the competing demands for its products and substrates *in vivo* and the connecting metabolic sequences utilizing these amine intermediates in the plant.

## Acknowledgement

The financial assistance received from University Grants Commission, New Delhi is gratefully acknowledged.

## References

- Adiga, P. R. and Prasad, G. L. (1985) *J. Plant Growth Regul.*, **3**, 203.
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.*, **121**, 404.
- Jencks, W. P. (1970) in *CRC Handbook of Biochemistry* (ed. H. A. Sober) (Ohio: CRC Press) J181.
- Laemmli, U. K. (1970) *Nature (London)*, **227**, 680.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Ouchterlony, O. (1967) in *Handbook of Experimental Immunology* (ed. D. M. Weir) (Oxford and Edinburgh: Blackwell Scientific Publications) p. 655.
- Prasad, G. L. and Adiga, P. R. (1985a) *J. Plant Growth Regul.*, **4**, 49.
- Prasad, G. L. and Adiga, P. R. (1985b) *J. Biosci.*, **7**, 331.
- Prasad, G. L. and Adiga, P. R. (1986a) *J. Biosci.*, **10**, 203.
- Prasad, G. L. and Adiga, P. R. (1986b) *J. Biosci.*, **10**, 373.
- Rabinovitch, B. S. and Flowers, M. C. Q. (1964) *Rev. Chem. Soc., (London)*, **18**, 122.
- Ramakrishna, S. and Adiga, P. R. (1975) *Eur. J. Biochem.*, **59**, 377.
- Schewizer, E. (1980) in *Multifunctional Proteins* (eds H. Bisswanger and E. Schmincke-Ott) (Wiley-Interscience) p. 197.
- Schmit, J. C. and Zalkin, H. (1969) *Biochemistry*, **8**, 174.
- Srivenugopal, K. S. and Adiga, P. R. (1981) *J. Biol. Chem.*, **256**, 9532.
- Suresh, M. R., Ramakrishna, S. and Adiga, P. R. (1978) *Phytochemistry*, **17**, 57.
- Vance, D. E. (1976) *J. Ther. Biol.*, **59**, 409.
- Wakil, S. J., Stoops, J. K. and Joshi, V. C. (1983) *Annu. Rev. Biochem.*, **52**, 537.
- Welch, R. G. and Gaertner, F. H. (1980) *Curr. Topic Cell Regul.*, **16**, 113.



# Journal of Biosciences

Vol. 11, March 1987

## SUBJECT INDEX

- Accessibility  
 Reactivity of glycoconjugate in membrane system II: can a neutral glycolipid function as lectin receptor in the presence of gangliosides in plasma membrane? 495
- Acetoacetate metabolism  
 Mode of action of lipoic acid in diabetes 59
- Acetylcholinesterase  
 Study of *Salmonella* endotoxin on the changes in lipid-protein interactions of membranes using Arrhenius plots of acetylcholinesterase as a tool 81
- Acidic vesicles  
 How does cobalamin (vitamin B12) enter and traverse mammalian cells? 75
- Acyl-CoA  
 Alteration of the acyl chain composition of free fatty acids, acyl coenzyme A and other lipids by dietary polyunsaturated fats 443
- Affinity chromatography  
 Transcriptional specificity after mycobacteriophage I3 infection 167
- Agrobacterium rhizogenes*  
 Molecular biology of Ri-plasmid—A review 47
- Alkali  
 Effect of alkaline pH on sunflower 11S protein 351
- 6-Aminopenicillanic acid  
 Immobilization of *Kluyvera citrophila* penicillin acylase on controlled-pore ceramics 549
- Amylase  
 Multisubstrate specific amylase from mushroom *Termitomyces clypeatus* 275
- Antielastase activity  
 Are the proteinase inhibitory activities in lenticular tissues real? 321
- Antitryptic activity  
 Are the proteinase inhibitory activities in lenticular tissues real? 321
- Aphidicolin inhibition  
 Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase- $\alpha$  from embryonic chicken brain 361
- Arginine decarboxylase  
 Arginine decarboxylase is a component activity of the multifunctional enzyme, putrescine
- Autoregulation  
 Autoregulation of gene expression: *rho* 225
- Avirulent strain  
 Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of *Vibrio cholerae* 231
- Bengal gram  
 The positional specificities of the oxygenation of linoleic acid catalyzed by two forms of lipoxygenase isolated from Bengal gram (*Cicer arietinum*) 527
- Bilirubin-amine interactions  
 Spectroscopic studies on the interaction of bilirubin with symmetrical alkyl diamines 485
- Bilirubin conformations  
 Spectroscopic studies on the interaction of bilirubin with symmetrical alkyl diamines 485
- Binding constants  
 Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to *Vicia faba* lectin: Fluorescence-quenching studies 537
- Binding-site amino acids  
 Physicochemical properties and binding-site amino acid residues of galactoside-binding protein of human placenta 311
- Biological role  
 A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver 379
- Blood substitute  
 Metabolism of an isolated brain perfused with perfluoro blood substitute 23
- Brain metabolism  
 Metabolism of an isolated brain perfused with perfluoro blood substitute 23
- Brain tumors  
 Antigenic components of human glioblastoma multiforme and rat C<sub>6</sub> glioma using monoclonal antibodies 423
- B-ring  
 Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets 525



Cells	
How does cobalamin (vitamin B12) enter and traverse mammalian cells?	75
Cell surface	
Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblast cells induced by glucocorticoids	107
Chemical modification	
Physicochemical properties and binding-site amino acid residues of galactosides-binding protein of human placenta	311
Effect of chemical modification on structure and activity of glucoamylase from <i>Aspergillus candidus</i> and <i>Rhizopus</i> species	339
Chicken liver	
A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver	379
Chronic myeloid leukaemia	
Abnormal erythrocyte membrane phospholipids organisation in chronic myeloid leukaemia	543
Cibacron Blue F3G-A	
Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase	265
Cis and trans unsaturated fatty acids	
Effect of cis and trans unsaturated fatty acids on the transport properties of <i>Salmonella typhimurium</i>	145
Cobalamin	
How does cobalamin (vitamin B12) enter and traverse mammalian cells?	75
Coenzyme A	
Mode of action of lipoic acid in diabetes	59
Coimmobilization	
Coimmobilization of D-amino acid oxidase and catalase by entrapment of <i>Trigonopsis variabilis</i> in radiation polymerised polyacrylamide beads	137
Colchicine-tubulin dimer complex	
Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets	525
Conformation	
Conformational change of L7/L12 stalk in the different functional states of 50S ribosomes	561
Conjugative F-plasmid	
Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein via conjugative plasmid F in <i>Escherichia coli</i> K-12	181
Controlled-pore ceramics	
Immobilization of <i>Kluyvera citrophila</i> penicillin acylase on controlled-pore ceramics	549
Cyclic AMP-receptor protein complex	
conjugative plasmid F in <i>Escherichia coli</i> K-12	181
Cytochrome P-450	
Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450	35
D-amino acid oxidase	
Coimmobilization of D-amino acid oxidase and catalase by entrapment of <i>Trigonopsis variabilis</i> in radiation polymerised polyacrylamide beads	137
1-D and 2-D NMR of peptides	
Conformational and ion-binding properties of cyclolinopeptide A isolated from linseed	473
Denaturation	
Effect of alkaline pH on sunflower 11S protein	351
Diabetes	
Mode of action of lipoic acid in diabetes	59
Diethylhexyl phthalate	
Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat	391
Difference spectroscopy	
Spectroscopic studies on the interaction of bilirubin with symmetrical alkyl diamines	485
Differential scanning calorimetry	
Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma	299
Dissociation	
Effect of alkaline pH on sunflower 11S protein	351
Distribution in membrane	
Reactivity of glycoconjugate in membrane system II: can a neutral glycolipid function as lectin receptor in the presence of gangliosides in plasma membrane?	495
DNA alkylation	
Purification and some properties of human DNA-O <sup>6</sup> -methylguanine methyltransferase	215
DNA chain initiation	
Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase- $\alpha$ from embryonic chicken brain	361
DNA polymerase- $\alpha$	
Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase- $\alpha$ from embryonic chicken brain	361
DNA repair	
Purification and some properties of human DNA-O <sup>6</sup> -methylguanine methyltransferase	215
Dolichyl monophosphate	
Amphomycin: A tool to study protein N-glycosylation	311
<i>Drosophila melanogaster</i>	
High affinity uptake of L-glutamate and $\gamma$ -	

Egg yolk plasma lipids	
Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma	299
Electrolytes	
Glycosaminoglycans in human fetal liver in relation to water and electrolytes	409
Endocytosis	
How does cobalamin (vitamin B12) enter and traverse mammalian cells?	75
Endogenous T-DNA	
Molecular biology of Ri-plasmid—A review	47
Endotoxin	
Study of <i>Salmonella</i> endotoxin on the changes in lipid-protein interactions of membranes using Arrhenius plots of acetylcholinesterase as a tool	81
Enzyme osmometry	
Definition of physical integrity of synaptosomes and myelosomes by enzyme osmometry	89
Erythrocytes	
Abnormal erythrocyte membrane phospholipids organisation in chronic myeloid leukaemia	543
<i>Escherichia coli</i> fimbriae	
Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein <i>via</i> conjugative plasmid F in <i>Escherichia coli</i> K-12	181
Essentiality of Lys-166	
Site-directed mutagenesis to determine essential residues of ribulose-bisphosphate carboxylase of <i>Rhodospirillum rubrum</i>	203
Essential tyrosine	
Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity	287
Fatty acid auxotroph	
Effect of <i>cis</i> and <i>trans</i> unsaturated fatty acids on the transport properties of <i>Salmonella typhimurium</i>	145
Fimbriation under cyclic AMP control	
Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein <i>via</i> conjugative plasmid F in <i>Escherichia coli</i> K-12	181
Fimbriation under F-plasmid control	
Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein <i>via</i> conjugative plasmid F in <i>Escherichia coli</i> K-12	181
Fluorescence	
Spectroscopic studies on the interaction of bilirubin with symmetrical alkyl diamines	485
Fluorescence energy transfer	
Fluorescence-quenching	
Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to <i>Vicia faba</i> lectin: Fluorescence-quenching studies	537
GABA	
High affinity uptake of L-glutamate and $\gamma$ -aminobutyric acid in <i>Drosophila melanogaster</i>	119
Galactose-binding lectin	
Physicochemical properties and binding-site amino acid residues of galactoside-binding protein of human placenta	311
Ganglioside	
Reactivity of glycoconjugate in membrane system II: can a neutral glycolipid function as lectin in receptor in the presence of gangliosides in plasma membrane?	495
Genetic disorders	
Mammalian sulfoconjugate metabolism	7
Glucoamylases	
Effect of chemical modification on structure and activity of glucoamylase from <i>Aspergillus candidus</i> and <i>Rhizopus</i> species	339
Glucocorticoids	
Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblasts cells induced by glucocorticoids	107
Glutamate	
High affinity uptake of L-glutamate and $\gamma$ -aminobutyric acid in <i>Drosophila melanogaster</i>	119
Glycoconjugates	
Oligosaccharide structure determination of glycoconjugates using lectins	41
Glycosaminoglycans	
Glycosaminoglycans in human fetal liver in relation to water and electrolytes	409
Histochemical localization of protein-polysaccharides in renal tissue	245
Glycosylation inhibitor	
Amphotycin: A tool to study protein N-glycosylation	311
Gossypol	
<i>In vitro</i> effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases	465
Growth factors	
Effect of mitogenic factors extracted from fetal lung fibroblasts on the <i>in vitro</i> growth of melanocytes obtained from normal and vitiligo subjects	399
Growth inhibition	
Cell surface and other morphological changes accompanying growth inhibition in simian virus	

Histochemistry	
Histochemical localization of protein-polysaccharides in renal tissue	245
Human fetuses	
Glycosaminoglycans in human fetal liver in relation to water and electrolytes	409
Human methyltransferase	
Purification and some properties of human DNA-O <sup>6</sup> -methylguanine methyltransferase	215
Human placenta	
Physicochemical properties and binding-site amino acid residues of galactoside-binding protein of human placenta	331
Human vitiligo	
Effect of mitogenic factors extracted from fetal lung fibroblasts on the <i>in vitro</i> growth of melanocytes obtained from normal and vitiligo subjects	399
<i>Humicola lanuginosa</i>	
Stabilisation of some of the protein synthesis components in the thermophilic fungus, <i>Humicola lanuginosa</i>	193
Hydroperoxides	
Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450	35
Hydroxylation reactions	
Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450	35
Increase	
Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat	391
Inhibition	
Study of <i>Salmonella</i> endotoxin on the changes in lipid-protein interactions of membranes using Arrhenius plots of acetylcholinesterase as a tool	81
Immobilization	
Immobilization of <i>Kluyvera citrophila</i> penicillin acylase on controlled-pore ceramics	549
Ionophoretic assay	
Conformational and ion-binding properties of cyclinopeptide A isolated from linsed	473
Isometallothionein	
A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver	379
Kidney	
Histochemical localization of protein-polysaccharides in renal tissue	245
<i>Kluyvera citrophila</i>	
Immobilization of <i>Kluyvera citrophila</i> penicillin acylase on controlled-pore ceramics	549
Lactic dehydrogenase-X	
<i>In vitro</i> effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases	465
Lanthanide probes	
Conformational and ion-binding properties of cyclinopeptide A isolated from linseed	473
Lectin receptor	
Reactivity of glycoconjugate in membrane system II: can a neutral glycolipid function as lectin receptor in the presence of gangliosides in plasma membrane?	495
Lectins	
Oligosaccharide structure determination of glycoconjugates using lectins	41
Lectin-saccharide binding	
Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to <i>Vicia faba</i> lectin: Fluorescence-quenching studies	537
Lenticular tissue	
Are the proteinase inhibitory activities in lenticular tissues real?	321
Lipid biosynthesis	
Mode of action of lipoic acid in diabetes	59
Lipid-linked pathway	
Amphomycin: A tool to study protein N-glycosylation	311
Lipid peroxidation	
Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450	35
Lipids	
Alteration of the acyl chain composition of free fatty acids, acyl coenzyme A and other lipids by dietary polyunsaturated fats	443
Lipoic acid	
Mode of action of lipoic acid in diabetes	59
Lipoxygenase	
The positional specificities of the oxygenation of linoleic acid catalyzed by two forms of lipoxygenase isolated from Bengal gram ( <i>Cicer arietinum</i> )	527
Liver	
Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat	391
Macrophages	
Importance of determining viability of <i>Mycobacterium leprae</i> inside macrophages—an <i>in vitro</i> method using uracil	455
Malate dehydrogenase	
<i>In vitro</i> effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases	465
Melanocyte culture	
Effect of mitogenic factors extracted from fetal	

## Membrane

- Study of *Salmonella* endotoxin on the changes in lipid-protein interactions of membranes using Arrhenius plots of acetylcholinesterase as a tool 81

## Metallothionein

- A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver 379

## 5,10-Methylenetetrahydrofolate

- Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase 265

## O<sup>6</sup>-Methylguanine

- Purification and some properties of human DNA-O<sup>6</sup>-methylguanine methyltransferase 215

## Modified RNA polymerase

- Transcriptional specificity after mycobacteriophage I3 infection 167

## Monoclonal antibodies

- Antigenic components of human glioblastoma multiforme and rat C<sub>6</sub> glioma using monoclonal antibodies 423

## Morphological changes

- Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblasts cells induced by glucocorticoids 107

## Multifunctional enzyme

- Arginine decarboxylase is a component activity of the multifunctional enzyme putrescine synthase in cucumber seedlings 571

## *Mycobacterium leprae*

- Importance of determining viability of *Mycobacterium leprae* inside macrophages—an *in vitro* method using uracil 455

## *Naja naja*

- Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity 287

## N-bromosuccinimide oxidation

- Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity 287

## Neonatal

- A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver 379

## Neurotransmitter uptake

- High affinity uptake of L-glutamate and  $\gamma$ -aminobutyric acid in *Drosophila melanogaster* 119

## Non-essentiality of His-291

- fatty acids, acyl coenzyme A and other lipids by dietary polyunsaturated fats 443  
2',5'-OligoA synthetase activities  
RNase F and 2',5'-oligoA synthetase activities in mice after poly(I).poly(C) administration 239

## Oligosaccharide structures

- Oligosaccharide structure determination of glycoconjugates using lectins 41

## Ontogenic-regulation

- A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver 379

## Osmolytes

- Definition of physical integrity of synaptosomes and myelosomes by enzyme osmometry 89

## Oxygen radicals

- Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450 35

## Penicillin acylase

- Immobilization of *Kluyvera citrophila* penicillin acylase on controlled-pore ceramics 549

## Peptide chain initiation factor

- Mechanism of protein synthesis inhibition by vaccinia viral core and reversal of this inhibition by reticulocyte peptide chain initiation factors 503

## Perfluorochemicals

- Metabolism of an isolated brain perfused with perfluoro blood substitute 23

## Perfused brain

- Metabolism of an isolated brain perfused with perfluoro blood substitute 23

## Peroxides

- Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450 35

## Phase state asymmetry

- Abnormal erythrocyte membrane phospholipids organisation in chronic myeloid leukaemia 543

## Phospholipid asymmetry

- Abnormal erythrocyte membrane phospholipids organisation in chronic myeloid leukaemia 543

## Polyacrylamide beads

- Coimmobilization of D-amino acid oxidase and catalase by entrapment of *Trigonopsis variabilis* in radiation polymerised polyacrylamide beads 137

## Poly(I).Poly(C) administration

- RNase F and 2',5'-oligoA synthetase activities in mice after poly(I).poly(C) administration 239

## Polysaccharidase

- Multisubstrate specific amylase from mushroom 276

- Primase N-ethylmaleimide inhibition  
Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase- $\alpha$  from embryonic chicken brain 361
- Procion Red HE-3B  
Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase 265
- Proteinase inhibitor  
Are the proteinase inhibitory activities in lenticular tissues real? 321
- Protein N-glycosylation  
Amphomycin: A tool to study protein N-glycosylation 311
- Protein synthesis  
Mechanism of protein synthesis inhibition by vaccinia viral core and reversal of this inhibition by reticulocyte peptide chain initiation factors 503  
Conformational change of L7/L12 stalk in the different functional states of 50S ribosomes 561
- Protoplast stabilization  
Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity 287
- Putrescine biosynthesis  
Arginine decarboxylase is a component activity of the multifunctional enzyme putrescine synthase in cucumber seedlings 571
- Pyruvate dehydrogenase  
Mode of action of lipoic acid in diabetes 59
- Rate constant  
Scavenging of superoxide radical by ascorbic acid 435
- Rate of protein turnover  
Stabilisation of some of the protein synthesis components in the thermophilic fungus, *Humicola lanuginosa* 193
- Receptor  
How does cobalamin (vitamin B12) enter and traverse mammalian cells? 75
- Regulation  
Effect of proteolytic digestion on the function of vesicular stomatitis virus ribonucleoprotein complex 515
- Rho* gene  
Autoregulation of gene expression: *rho* 225
- Ribosomes  
Conformational change of L7/L12 stalk in the different functional states of 50S ribosomes 561
- Ribulose- $P_2$  carboxylase  
Rifampicin-Sepharose  
Transcriptional specificity after mycobacteriophage I3 infection 167
- Ri-plasmid  
Molecular biology of Ri-plasmid—A review 47
- RNA nucleotidyl transferase  
Generation of bacteriophage T3 mRNAs by post-transcriptional processing by RNase III 155
- RNA polymerase  
Transcriptional specificity after mycobacteriophage I3 infection 167
- RNA processing  
Generation of bacteriophage T3 mRNAs by post-transcriptional processing by RNase III 155
- RNase III  
Generation of bacteriophage T3 mRNAs by post-transcriptional processing by RNase III 155
- RNase F  
RNase F and 2',5'-oligoA synthetase activities in mice after poly(I).poly(C) administration 239
- Simian virus  
Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblasts cells induced by glucocorticoids 107
- Site-directed mutagenesis  
Site-directed mutagenesis to determine essential residues of ribulose-bisphosphate carboxylase of *Rhodospirillum rubrum* 203
- Stabilisation of synthetase  
Stabilisation of some of the protein synthesis components in the thermophilic fungus, *Humicola lanuginosa* 193
- Structure and activity  
Effect of chemical modification on structure and activity of glucoamylase from *Aspergillus candidus* and *Rhizopus* species 339
- Sulfatases  
Mammalian sulfoconjugate metabolism 7
- Sulfoconjugates  
Mammalian sulfoconjugate metabolism 7
- Sulfotransferases  
Mammalian sulfoconjugate metabolism 7
- Sunflower 11S protein  
Effect of alkaline pH on sunflower 11S protein 351
- Superoxide dismutase  
Scavenging of superoxide radical by ascorbic acid 435

tion of diethylhexyl phthalate to the rat	391	and myelosomes by enzyme osmometry	89
<i>Termitomyces clypeatus</i>		$\beta$ -Turn and calcium ion binding	
Multisubstrate specific amylase from mushroom		Conformational and ion-binding properties of cyclolinopeptide A isolated from linseed	473
<i>Termitomyces clypeatus</i>	275	Type 1 fimbriation	
Testicular dehydrogenases		Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein via conjugative plasmid F in <i>Escherichia coli</i> K-12	181
<i>In vitro</i> effects of gossypol on testicular lactic dehydrogenase-X and other dehydrogenases	465	Ubiquinone	
Thermophilic fungus		Increase in hepatic ubiquinone on administration of diethylhexyl phthalate to the rat	391
Stabilisation of some of the protein synthesis components in the thermophilic fungus, <i>Humicola lanuginosa</i>	193	Uptake	
Thiocyanate space		How does cobalamin (vitamin B12) enter and traverse mammalian cells?	75
Glycosaminoglycans in human fetal liver in relation to water and electrolytes	409	Uracil uptake	
3T3 mouse fibroblasts cells		Importance of determining viability of <i>Mycobacterium leprae</i> inside macrophages—an <i>in vitro</i> method using uracil	455
Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblasts cells induced by glucocorticoids	107	Vaccinia virus	
Toxin gene		Mechanism of protein synthesis inhibition by vaccinia viral core and reversal of this inhibition by reticulocyte peptide chain initiation factors	503
Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of <i>Vibrio cholerae</i>	231	Very low density lipoproteins	
T3 promoter		Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma	299
Generation of bacteriophage T3 mRNAs by post-transcriptional processing by RNase III	155	Vesicular stomatitis virus	
Transcription		Effect of proteolytic digestion on the function of vesicular stomatitis virus ribonucleoprotein complex	515
Effect of proteolytic digestion on the function of vesicular stomatitis virus ribonucleoprotein complex	515	Viability assay	
Transcriptional control		Importance of determining viability of <i>Mycobacterium leprae</i> inside macrophages—an <i>in vitro</i> method using uracil	455
Transcriptional specificity after mycobacteriophage I3 infection	167	<i>Vibrio cholerae</i>	
Transition temperature		Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of <i>Vibrio cholerae</i>	231
Study of <i>Salmonella</i> endotoxin on the changes in lipid-protein interactions of membranes using arrhenius plots of acetylcholinesterase as a tool	81	<i>Vicia faba</i> lectin	
Transcription termination		Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to <i>Vicia faba</i> lectin: Fluorescence-quenching studies	537
Autoregulation of gene expression: <i>rho</i>	225	Xylanase	
Transport		Multisubstrate specific amylase from mushroom <i>Termitomyces clypeatus</i>	275
Effect of <i>cis</i> and <i>trans</i> unsaturated fatty acids on the transport properties of <i>Salmonella typhimurium</i>	145	Zinc	
<i>Trigonopsis variabilis</i>		A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver	379
Coimmobilization of D-amino acid oxidase and catalase by entrapment of <i>Trigonopsis variabilis</i> in radiation polymerised polyacrylamide beads	137		
Tubulin-sheets			
Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets	525		



## AUTHOR INDEX

- Charya, S. R.  
 see Ramarao, C. S. 119  
 dhya, Sankar  
 see Haber, Roberta 225  
 diga, P. R.  
 see Prasad, G. L. 571  
 dhya, Samit  
 see Majumder, Hemanta K. 155  
 garwal, S. S.  
 see Kumar, A. 543  
 nuradha Nandi  
 Scavenging of superoxide radical by ascorbic acid 435  
 opukuttan, P. S.-  
 see Basu, Debkumar 41  
 see Nambiar, Madhusoodhan P. 331  
 shwini Deshpande  
 Coimmobilization of D-amino acid oxidase and catalase by entrapment of *Trigonopsis variabilis* in radiation polymerised polyacrylamide beads 137  
 ngchi, Milan  
 see Chakrabarti, Debopam 503  
 ksi, Krishna  
 RNase F and 2',5'-oligoA synthetase activities in mice after poly(I).poly(C) administration 239  
 laram, P.  
 see Purnam, Kasturi Lahiri 485  
 lasubramanian, A. S.  
 see Mathew, Joy 7  
 lasubramanian, D.  
 see Chatterji, D. 473  
 mji, Mahtab S.  
 see Giridharan, N. 465  
 nerjee, Asok  
 Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets 525  
 nerjee, A. K.  
 Effect of proteolytic digestion on the function of vesicular stomatitis virus ribonucleoprotein complex 515  
 nerjee, Dipak Kumar  
 Amphomycin: A tool to study protein N-glycosylation 311  
 ratz, Robert  
 see Das, Hasi R. 107  
 su, Debkumar  
 Oligosaccharide structure determination of glycoconjugates using lectins 41  
 Basu, Pranab S.  
 see Datta, Pradip K. 537  
 Basu, Subhash  
 see Ira Simet 361  
 Bhat, B. Ganesh  
 see Borthakur, Alip 257  
 Bhattacharyya, B  
 see Banerjee, Asok 525  
 Biswas, B. B.  
 see Chakraborty, T 379  
 Bodhe, A. M.  
 Immobilization of *Kluyvera citrophila* penicillin acylase on controlled-pore ceramics 549  
 Borthakur, Alip  
 The positional specificities of the oxygenation of linoleic acid catalyzed by two forms of lipoxigenase isolated from Bengal gram (*Cicer arietinum*) 257  
 Boulden, Amy M.  
 Purification and some properties of human DNA-O<sup>6</sup>-methylguanine methyltransferase 215  
 Brenda, Culp  
 see Prasad, M. Renuka 443  
 Burma, Debi P.  
 see Dash, Debabrata 561  
 Chakrabarti, Debopam  
 Mechanism of protein synthesis inhibition by vaccinia viral core and reversal of this inhibition by reticulocyte peptide chain initiation factors 503  
 Chakraborti, A.  
 see Podder, S. K. 495  
 Chakraborty, T.  
 A single form of metallothionein is present in both heavy metal induced and neonatal chicken liver 379  
 Chakravorty, M.  
 see Deb, J. K. 145  
 Chameli Ganguly  
 Glycosaminoglycans in human fetal liver in relation to water and electrolytes 409  
 Chatterjee, G. C.  
 see Ray, M. K. 81  
 Chatterjee, I. B.  
 see Anuradha Nandi 435  
 Chatterji, D.  
 Conformational and ion-binding properties of



Chattopadhyay, D. J. see Banerjee, A. K.	515	Gordon, Milton P. see Sinkar, Vilas P.	47
Chaudhuri, Sukla Ray see Banerjee, Asok	525	Goyal, R. see Deb, J. K.	145
Cherayil, Joseph D. see Joshi, Anil K.	193	Gupta, C. M. see Kumar, A.	543
Coon, Minor J. Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450	35	Gupta, Naba K. see Chakrabarti, Debopam	503
Daniel, S. see Kumar, A.	543	Haber, Roberta Autoregulation of gene expression: <i>rho</i>	225
Das, Hasi R. Cell surface and other morphological changes accompanying growth inhibition in simian virus 40-transformed 3T3 mouse fibroblasts cells induced by glucocorticoids	107	Hartman, Fred C. see Niyogi, Salil K.	203
Dash, Debabrata Conformational change of L7/L12 stalk in the different functional states of 50S ribosomes	561	Ira Simet Resolution of DNA polymerase- $\alpha$ -primase complex and primase free DNA polymerase- $\alpha$ from embryonic chicken brain	361
Datta, Pradip K. Binding of 4-methyl umbelliferyl- $\alpha$ -D-glucopyranoside to <i>Vicia faba</i> lectin: Fluorescence-quenching studies	537	Joshi, Anil K. Stabilisation of some of the protein synthesis components in the thermophilic fungus, <i>Humicola lanuginosa</i>	193
Datta, Tapash K. see Datta, Pradip K.	537	Jyoti, V. Nair see Basu, Deb Kumar	41
Deb, J. K. Effect of <i>cis</i> and <i>trans</i> unsaturated fatty acids on the transport properties of <i>Salmonella typhimurium</i>	145	Kenkare, U. W. see Ramarao, C. S.	119
D'Souza, S. F. see Ashwini, Deshpande	137	Kochhar, Kulvinder, S. see Ela Sharma	423
Ela Sharma Antigenic components of human glioblastoma multiforme and rat C <sub>6</sub> glioma using monoclonal antibodies	423	Krishnan, K. S. see Ramarao, C. S.	119
Eva H. Lee see Niyogi, Salil K.	203	Kumar, A. Abnormal erythrocyte membrane phospholipids organisation in chronic myeloid leukaemia	543
Fleming, Scott G. see Boulden, Amy M.	215	Kumar, Sushil see Shambhavi Subbarao	181
Foote, Robert S. see Niyogi, Salil K.	203	Kurup, C. K. Ramakrishna see Nandini Nair	391
see Boulden, Amy M.	215	Lands, William E. M. see Prasad, M. Renuka	443
Ghosh, Amit see Uma, Bhattacharyya	231	Larimer, Frank W. see Niyogi, Salil K.	203
Ghosh, Anil K. Multisubstrate specific amylases from mushroom <i>Termitomyces clypeatus</i>	275	Machanoff, Richard see Niyogi, Salil K.	203
Ghosh, Ranajit K. see Uma, Bhattacharyya	231	Madhyastha, M. N. see Giridharan, N.	465
Giridharan, N. <i>In vitro</i> effects of gossypol on testicular lactic acid dehydrogenase X-ray crystallographic studies	165	Mahadevan, S. Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma	299
		Mahadevan, P. R. see Sunanda, Vejare	455
		Mahanti, Subodh see Deb, J. K.	551

ity, Sankar N.		Prakash, Nam	
see Banerjee, Asok	525	see Shambhavi Subbarao	181
ujumder, Hemanta K.		Prasad, G. L.	
Generation of bacteriophage T3 mRNAs by		Arginine decarboxylase is a component activity	
post-transcriptional processing by RNase III	155	of the multifunctional enzyme putrescine	
		synthase in cucumber seedlings	571
y, Mathew		Prasad, M. Renuka	
Mammalian sulfoconjugate metabolism	7	Alteration of the acyl chain composition of free	
enon, K. K. G.		fatty acids, acyl coenzyme A and other lipids by	
see Wagh, S. S.	59	dietary polyunsaturated fats	443
itra, A		Purnam, Kasturi Lahiri	
see Ray, M. K.	81	Spectroscopic studies on the interaction of	
itra, Sankar		bilirubin with symmetrical alkyl diamines	485
see Niyogi, Salil K.	203		
see Boulden, Amy M.	215	Ramachandran, L. K.	
ojamdar, Manoj		see Shashidharan, P.	287
Effect of mitogenic factors extracted from fetal		Ramadoss, Candadai S.	
lung fibroblasts on the <i>in vitro</i> growth of		see Borthakur, Alip	257
melanocytes obtained from normal and vitiligo		Ramaiah, Abburi	
subjects	399	see Mojamdar, Manoj	399
ukherjee, B.		Ramaiah, Kolluru V. A.	
see Ray, M. K.	81	see Chakrabarti, Debopam	503
ukherjee, K. L.		Ramarao, C. S.	
see Chameli Ganguly	409	High affinity uptake of L-glutamate and $\gamma$ -	
ukherji, B.		aminobutyric acid in <i>Drosophila melanogaster</i>	119
Metabolism of an isolated brain perfused with			
perfluoro blood substitute	23	Rao, A. G. Appu	
ural, Richard J.		see Shenoy, B. C.	339
see Niyogi, Salil K.	203	Rao, N. Appaji	
		see Varalakshmi, K.	265
adkarni, G. B.		Rao, Narasinga M. S.	
see Ashwini, Deshpande	137	see Sripad, G.	351
agaraja, V.		Rao, Raghavendra M. R.	
Transcriptional specificity after mycobacterio-		see Shenoy, B. C.	339
phage I3 infection	167	Ray, M. K.	
ambiar, Madhusoodhan P.		Study of <i>Salmonella</i> endotoxin on the changes in	
Physicochemical properties and binding-site		lipid-protein interactions of membranes using	
amino acid residues of galactoside-binding		arrhenius plots of acetylcholinesterase as a tool	81
protein of human placenta	331		
andini Nair		Ray, Satyajit	
Increase in hepatic ubiquinone on administra-		see Ira Simet	361
tion of diethylhexyl phthalate to the rat	391	Roy, Ananda L.	
atraj, C. V.		see Chakrabarti, Debopam	503
see Wagh, S. S.	59	Roy, J.	
celu, Puri		see Banerjee, A. K.	515
see Mojamdar, Manoj	399		
iyogi, Salil K.		Sankaram, M. B.	
Site-directed mutagenesis to determine essential		see Chatterji, D.	473
residues of ribulose-bisphosphate carboxylase of		Saraswathi, S.	
<i>Rhodospirillum rubrum</i>	203	Histochemical localization of protein-poly-	
		saccharides in renal tissue	245
attabiraman, T. N.		Sarma, M. K. Janardana	
see Swaminathan, S.	321	see Sitaramam, V.	89
odder, S. K.		Savithri, H. S.	
Reactivity of glycoconjugate in membrane		see Varalakshmi, K.	265
system II: can a neutral glucolipid function as		Seetharam Bellur	

Sengupta, Subhabrata see Ghosh, Anil K.	275	Sunanda, Vejare Importance of determining viability of <i>Mycobacterium leprae</i> inside macrophages—an <i>in vitro</i> method using uracil	455
Shail K. Sharma see Ela Sharma	423	Swaminathan, S. Are the proteinase inhibitory activities in lenticular tissues real?	321
Shambhavi Subbarao Type 1 fimbriation is negatively regulated by cyclic AMP and its receptor protein <i>via</i> conjugative plasmid F in <i>Escherichia coli</i> K-12	181	Tesoriero, J. V. see Saraswathi, S.	245
Shashidharan, P. Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity	287	Uma, Bhattacharyya Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of <i>Vibrio cholerae</i>	231
Shenoy, B. C. Effect of chemical modification on structure and activity of glucoamylase from <i>Aspergillus candidis</i> and <i>Rhizopus</i> species	339	Umadas, Maitra see Majumder, Hemanta K.	155
Singh, P. Lalit Kumar see Podder, S. K.	495	Varalakshmi, K. Interaction of Cibacron Blue F3G-A and Procion Red HE-3B with sheep liver 5,10-methylenetetrahydrofolate reductase	265
Sinkar, Vilas P. Molecular biology of Ri-plasmid—A review	47	Vasan, N. S. see Saraswathi, S.	245
Sitaramam, V. Definition of physical integrity of synaptosomes and myelosomes by enzyme osmometry	89	Vaz, Alfin D. N. see Coon, Minor J.	35
Sivaprasad, A. V. see Shambhavi Subbarao	181	Vijayalakshmi, K. see Podder, S. K.	495
SivaRaman, C. see Bodhe, A. M.	549	Wagh, S. S. Mode of action of lipoic acid in diabetes	59
Skurkovich, Boris see Baksi, Krishna	239	White, Frank F. see Sinkar, Vilas P.	47
Skurkovich, Simon see Baksi, Krishna	239	Young, Delano V. see Das, Hasi R.	107
Sloviter, H. A. see Mukherji, B.	23		
Soper, Thomas S. see Niyogi, Salil K.	203		
Sripad, G. Effect of alkaline pH on sunflower 11S protein	351		

